

Form PTO-1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>6460-18-1</b>	
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>				U.S. APPLICATION NO. (if known, see 37 CFR 1.51) <b>09/868469</b>	
INTERNATIONAL APPLICATION NO. PCT/US99/30162		INTERNATIONAL FILING DATE 18 December 1999		PRIORITY DATE CLAIMED 18 December 1998	
TITLE OF INVENTION CYCLIC PEPTIDES				<b>JG04 Rec'd PCT/PTO 15 JUN 2001</b>	
APPLICANT(S) FOR DO/EO/US BENKOVIC, Stephen J.; SCOTT, Charles P.; ABEL-SANTOS, Ernesto V.					
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</li> </ol> </li> <li>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (unsigned)</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p><b>Items 11. to 16. below concern document(s) or information included:</b></p> <ol style="list-style-type: none"> <li>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.  <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input checked="" type="checkbox"/> Other items or information: 2 return receipt postcards</li> </ol>					

EXPRESS MAIL LABEL NO. EL649720582US

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) **09/868469**

INTERNATIONAL APPLICATION NO.  
PCT/US99/30162

JC003 Rec'd PCT/PTC 15 JUN 2001  
ATTORNEY'S DOCKET NUMBER 6460-18-1

17. ☐ The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... **\$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... **\$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... **\$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... **\$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... **\$100.00**

**CALCULATIONS** PTO USE ONLY

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

**\$690.00**

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

**\$**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	127 -20 =	107	X <b>\$18.00</b>
Independent claims	11 -3 =	8	X <b>\$80.00</b>

**\$1926.00**

**\$640.00**

**MULTIPLE DEPENDENT CLAIM(S)** (if applicable)

**+ \$270.00**

**\$**

**TOTAL OF ABOVE CALCULATIONS =**

**\$3256.00**

Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

**\$1628.00**

**SUBTOTAL =**

**\$1628.00**

Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.429(f)).

**+ \$**

**TOTAL NATIONAL FEE =**

**\$1628.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **\$40.00** per property

**+ \$**

**TOTAL FEES ENCLOSED =**

**\$1628.00**

Amount to be:  
refunded **\$**

Charged **\$**

a. ☐ A check in the amount of \$     .00     to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 50-0951 in the amount of \$ 1628.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0951. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

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NAME

42,730

REGISTRATION NUMBER

CYCLIC PEPTIDESCross Reference To Related Applications

The present application claims the benefit of U.S. provisional patent application Serial No. 60/112,723 filed December 18, 1998 and U.S. provisional patent application Serial No. \_\_\_\_\_ entitled "Production of Cyclic Peptides and Proteins In Vivo" filed October 7, 1999, both of which are incorporated herein by reference.

Statement As To Federally-Sponsored Research

This invention was made with Government support under grants GM13306 and GM19891 awarded by the National Institutes of Health. The Government may have certain rights in the invention.

Field Of The Invention

The invention relates to the field of biochemistry. More particularly, the invention relates to cyclic peptides, methods for the making cyclic peptides, and methods of screening cyclic peptides for particular characteristics.

Background Of The Invention

Small linear peptides are useful for investigating various physiological phenomena because they exhibit a wide range of biological activities and can be easily synthesized in almost infinitely variable sequences utilizing conventional techniques in solid phase synthesis and combinatorial chemistry. These qualities also make small linear peptides especially useful for identifying and developing new drugs. For example, large libraries of myriad

different small linear peptides can be prepared synthetically and then screened for a particular characteristic in various biological assays. E.g., Scott, J. K. and G. P. Smith, Science 249:386, 1990; Devlin, J. J., et al., Science 24:404, 1990; Furka, A. et al., Int. J. Pept. Protein Res. 37:487, 1991; Lam, K. S., et al., Nature 354:82, 1991. Those peptides within the library that exhibit the particular characteristic can then be isolated as candidates for further study. Microsequencing or other chemical analyses can then be used to characterize selected peptides by, for example, amino acid sequence. Despite these advantages, only a handful of small linear peptides have been developed into widely-used pharmaceutical drugs. One reason for this is that small linear peptides are usually cleared from the body too rapidly to be of therapeutic value

Ring closure, or cyclization, can reduce the rate at which peptides are degraded in vivo and therefore dramatically improve their pharmacokinetic properties. The majority of cyclic peptides of known therapeutic value have been identified after isolation from natural sources (e.g., calcitonins, oxytocin, and vasopressin). Unfortunately, the pool of naturally-existing cyclic peptides that can be screened for a particular biological activity is inherently limited. And, moreover, the onerous steps required to isolate and purify cyclic peptides from natural sources render such screens costly and impractical. Thus, synthetic methods for producing large numbers of different peptides of infinitely variable amino acid sequences would greatly facilitate identifying particular cyclic peptides as candidates for new drugs.

Various methods for producing cyclic peptides have been described. For example, chemical reaction protocols, such as those described in U.S. Patent Nos. 4,033,940 and 4,102,877, have been devised to produce circularized peptides. In other techniques, biological and chemical methods are combined to produce cyclic peptides. These latter methods involve first expressing linear precursors of cyclic peptides in cells (e.g., bacteria) to

produce linear precursors of cyclic peptides and then adding of an exogenous agent such as a protease or a nucleophilic reagent to chemically convert these linear precursors into cyclic peptides. See, e.g., Camerero, J. A., and Muir, T. W., J. Am. Chem. Society. 121:5597 (1999); Wu, H. et al, Proc. Natl. Acad. Sci. USA, 95:9226 (1998).

5           Once produced, cyclic peptides can be screened for pharmacological activity. For example, a library containing large numbers of different cyclic peptides can be prepared and then screened for a particular characteristic, such as the ability to bind a specific target ligand. The library is mixed with the target ligand, and those members of the library that bind to the target ligand can be isolated and identified by amino acid sequencing. Similarly, libraries of cyclic peptides can be added to assays for a specific biological activity. Those cyclic peptides which modulate the biological activity can then be isolated and identified by sequencing.

10           Unfortunately, because the step of identifying the active peptides can be difficult, these screening assays can prove laborious and time-consuming. For instance, screening assays usually mandate a reverse-mapping step because the actual amount of cyclic peptide that binds a target ligand or modulates a biological activity is usually so minute that it cannot be sequenced directly. To avoid this problem, a map indicating the physical location of the various cyclic peptides comprising a library can be made. Aliquots of cyclic peptides from the different locations are then transferred to corresponding locations within the screening assay; and those areas in the assay that exhibit the screened-for activity (e.g., binding or  
15           modulation of biological activity) are then mapped back to their corresponding location in the library. The cyclic peptides in that area of the library can then be isolated and sequenced. Difficulties arising from the need for spatial resolution and the limitations imposed by sample handling limit the number of candidate peptides that can be screened in any given period of  
20           time.

The number of peptides that can be screened in an assay can be dramatically increased by using cells that express the peptides. For example, bacteria engineered to express a library of linear peptides can be added to a screening assay, and those bacteria that express the screened for characteristic can be picked directly from the assay. The picked bacteria can then be reproduced to large numbers such that the selected linear peptides can made in large quantities to facilitate their identification (e.g., by sequencing) and production. Making and screening small linear peptide libraries in vivo has, however, proven to be troublesome because small linear peptides are rapidly degraded by normal cellular metabolic processes. Cyclization of the peptides can circumvent this problem by rendering the peptides stable within a cell.

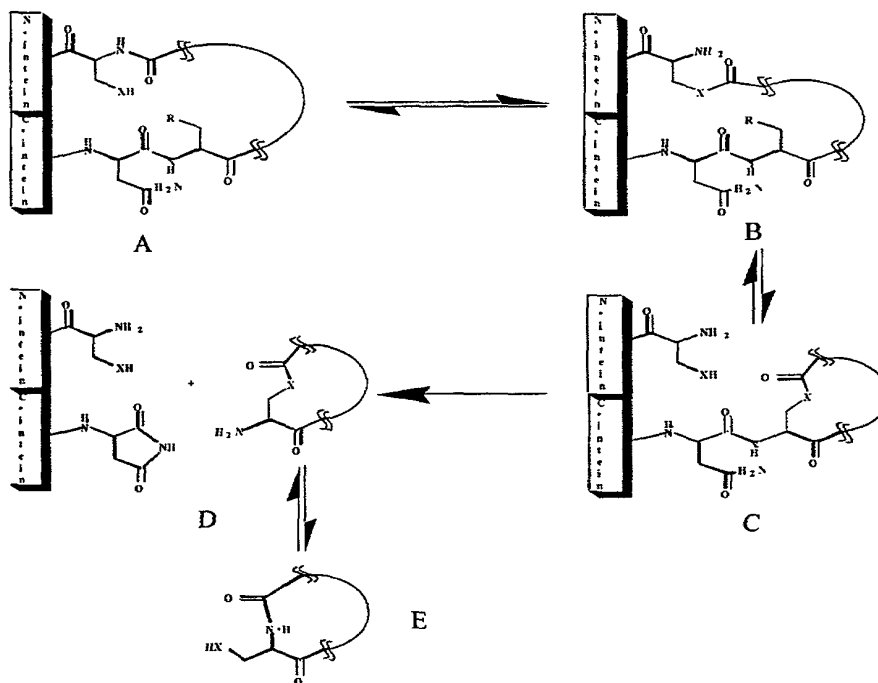
Despite this, heretofore, intracellular production of large libraries of cyclic peptides has not been feasible because general, easy-to-perform methods for cyclizing peptides in vivo have not been available. For example, a known method of producing cyclic peptides in vivo utilizes non-ribosomal peptide synthetase (NRPS) complexes (Cane et al, Science 282:63, 1998). Such NRSP complexes are, however, neither facile to work with nor generally useful for the production of more than a single cyclic peptide at a time. Moreover, unlike ribosomal peptide synthesis where the linear sequence of monomers (amino acids) is dictated by the linear sequence of bases in the nucleic acid molecule encoding it, the linear sequence of monomers in a peptide made by the NRPS method is dictated by the subunit organization of the NRPS complex. Changing the sequence of a cyclic peptide made by NRPS entails cloning the subunit(s) which incorporate the desired monomers and introducing the subunit(s) into host cells already harboring all of the other necessary subunits. Making a library using this technique would require introducing combinations (both in composition and order) of NRPS subunits to host cells and devising a method for ensuring that the subunits

assemble into the correct supramolecular structures.

### Summary Of The Invention

A general method for the in vivo production and screening of cyclic peptide libraries has been discovered. In this method, a nucleic acid molecule is constructed such that a nucleotide sequence encoding the peptide to be cyclized is flanked on one end with a nucleotide sequence encoding the carboxy-terminal portion of a split (or trans) intein (C-intein or  $I_C$ ) and on its other end with a nucleotide sequence encoding the amino-terminal portion of a split intein (N-intein or  $I_N$ ). Expression of the construct within a host system such as a bacterium or eukaryotic cell results in the production of a fusion protein. The two split intein components (i.e.,  $I_C$  and  $I_N$ ) of the fusion protein then assemble to form an active enzyme that splices the amino and carboxy termini together to generate a backbone cyclic peptide. The chemical reaction is depicted below.

#### Mechanism of Intein Mediated Cyclization



Formation of the active intein from the amino and carboxy-terminal fragments stabilizes the ester isomer of an amino acid at the junction between the N-intein and the peptide to be cyclized (in B above, X=S or O). When R=XH, the heteroatom from the C-intein is poised to attack the ester and generate a cyclic ester intermediate (C). Intein-catalyzed aminosuccinimide formation (D) liberates the cyclic peptide (in the lactone form), which spontaneously rearranges to form the thermodynamically favored backbone (lactam form) cyclic peptide product (E). This method can be adapted to facilitate the selection or screening of cyclic peptides with predetermined characteristics.

Accordingly the invention features a non-naturally occurring nucleic acid molecule encoding a polypeptide having a first portion of a split intein, a second portion of a split intein, and a target peptide interposed between the first portion of a split intein and the second portion of a split intein. Expression of the nucleic acid molecule in a host system produces a polypeptide that spontaneously splices in the host system to yield a cyclized form of the target peptide, or a splicing intermediate of a cyclized form of the target peptide such as an active intein intermediate, a thioester intermediate, or a lariat intermediate.

Both the first portion of a split intein and the second portion of a split intein can be derived from a naturally-occurring split intein such as Ssp DnaE. In other variations, one or both of split intein portions can be derived from non-naturally occurring split inteins such as those derived from RecA, DnaB, Psp Pol-I, and Pfu inteins.

In another aspect, the invention features a non-naturally occurring nucleic acid molecule encoding a polypeptide having a first portion of a split intein, a second portion of a split intein, a third portion of a split intein, and fourth portion of a split intein. This molecule can have a first target peptide is interposed between the first portion of a split intein and the second portion of a split intein, and a second target peptide is interposed between the third



portion of a split intein and the fourth portion of a split intein. The first portion of a split intein can be complementary to the third portion of a split intein but not complementary to the second portion of a split intein, and the second portion of a split intein can be complementary to the fourth portion of a split intein but not complementary to the third portion of a split intein.

Also within the invention is an expression vector comprising a nucleic acid molecule within the invention. Expression of the vector in a host system produces a polypeptide that spontaneously splices in the host system to yield a cyclic peptide or a splicing intermediate. The expression vector of the invention can also contain a regulatory sequence that facilitates expression of the polypeptide in the host system. The nucleic acid molecule of the vector can include a nucleotide sequence that encodes a peptide that facilitates screening of the cyclized form of the target peptide for a particular characteristic and/or a nucleotide sequence that encodes a peptide that facilitates purifying the cyclized form of the target peptide from the host system. The expression vector can also be inducible.

In another aspect, the invention features an expression vector encoding a polypeptide having a target peptide that has a first end fused to a first portion of a split intein and a second end fused to a second portion of a split intein. Expression vectors of the invention can be a plasmid, a bacteriophage, a virus, a linear nucleic acid molecule, or other type of vector.

The invention additionally features a substantially pure polypeptide having a first portion of a split intein, an second portion of a split intein, and a target peptide interposed between the first portion of a split intein and the second portion of a split intein. The polypeptide can be one that spontaneously splices in the host system to yield a cyclized form of the target peptide, or it can be a splicing intermediate.

Also within the invention is a host system harboring a nucleic acid molecule of the

invention. The host system can be a prokaryote such as a bacterium, an archaeobacterium, a eukaryote such as a yeast or a mammalian cell, a plant cell, an in vitro transcription/translation system, or a cell lysate.

In another aspect, the invention features a method for making a peptide molecule.

5 This method includes the steps of: providing an isolated nucleic acid molecule of the invention; providing a host system; introducing the isolated nucleic acid molecule into the host system; and expressing the isolated nucleic acid molecule. In one variation, the step of expressing the isolated nucleic acid molecule results in production of a polypeptide that spontaneously splices in the host system to yield the cyclized form of the target peptide. This method can also feature the step of purifying the cyclized form of the target peptide from the host system.

10 In another variation of this method, the step of expressing the isolated nucleic acid molecule results in production of a splicing intermediate of a cyclized form of the target peptide. This method can also feature the step of purifying the splicing intermediate of a cyclized form of the target peptide from the host system. Yet another variation of this method, includes the step of forming the cyclic peptide from the splicing intermediate.

In another aspect of this method, the target peptide is produced in a cyclized form in the host system in the absence of an exogenously-added agent such as a protease or a thiol.

15 Another aspect of the invention is a method of preparing a library of peptide molecules. This method involves the steps of providing a plurality of nucleic acid molecules encoding a plurality of target peptides having heterogenous amino acid sequences; incorporating each of the plurality of nucleic acid molecules into an expression vector to form a plurality of expression vectors, and expressing the expression vectors in the host system. The plurality of nucleic acid molecules is interposed between a nucleic acid molecule

encoding a first portion of a split intein and a nucleic acid molecule encoding an second portion of a split intein in each of the formed expression vectors such that expression of the expression vectors in a host system results in the production of a plurality of peptide molecules such as polypeptides that spontaneously splice in the host system to yield cyclized forms of the target peptides, or splicing intermediates of cyclized forms of the target peptides

And in yet another aspect, the invention includes a method of screening a peptide molecule for a predetermined characteristic. This method includes the steps of: providing a nucleic acid molecule that encodes a polypeptide comprising a first portion of a split intein, a second portion of a split intein, and a target peptide interposed between the first portion of a split intein and the second portion of a split intein; providing the host system; introducing the isolated nucleic acid molecule in the host system; placing the host system under conditions that cause the peptide molecule to be produced; and testing the peptide molecule for the predetermined characteristic. Expression of the nucleic acid molecule in a host system produces either a cyclized form of the target peptide resulting from spontaneously splicing of the polypeptide in the host system, or a splicing intermediate of a cyclized form of the target peptide.

In one variation of this method, the predetermined characteristic includes the ability to specifically bind a target molecule, and the step of testing the peptide molecule for the predetermined characteristic includes the steps of (a) contacting the peptide molecule to the target molecule and (b) determining whether the peptide molecule binds to the target molecule. In another variation, the predetermined characteristic is the ability to modulate a biochemical reaction, and the step of testing the peptide molecule for the predetermined characteristic comprises the steps of (a) contacting the peptide molecule to a system containing the biochemical reaction and (b) determining whether the peptide molecule

modulates the biochemical reaction. The step of determining whether the peptide molecule binds to a target molecule or modulates a biochemical reaction can be measured by observing a color change, a fluorescent signal, by analyzing the cell cycle or the reproduction of an organism.

5           The target molecule in these methods can be a cell-associated molecule such as a membrane-associated molecule or an intracellular molecule (e.g., a nuclear molecule or one or more organelles such as mitochondria, lysosomes, endoplasmic reticula, chloroplasts, golgi, and periplasm). It can also be an extracellular molecule.

10           The biochemical reaction can be a cell associated-process such as an intracellular metabolic event, a membrane-associated event, a nuclear event. It can also be an extracellular reaction.

15           In these methods, the step of testing the peptide molecule for the predetermined characteristic can be performed using a hybrid system, and/or the step of immobilizing the peptide molecule on a solid phase support.

20           The invention also features a method for purifying a cyclic peptide from a mixture. This method includes the steps of: providing a mixture containing a splicing intermediate conjugated with an affinity tag; mixing the conjugated splicing intermediate with a solid phase support having a ligand thereon that specifically binds the affinity tag such that the support becomes specifically bound with the splicing intermediate; washing the support to  
25           remove non-specifically bound matter from the support; adding to the support a reagent that makes a cyclic peptide from the splicing intermediate; and eluting the cyclic peptide from the support.

30           In a variation of the foregoing, the invention also includes a method for purifying a cyclic peptide from a mixture that includes the steps of: providing a mixture containing a

splicing intermediate conjugated with an affinity tag; mixing the conjugated splicing intermediate with a solid phase support having a ligand thereon that specifically binds the affinity tag such that the support becomes specifically bound with the splicing intermediate; washing the support to remove non-specifically bound matter from the support; eluting the splicing intermediate from the support; and adding a reagent the eluted splicing intermediate that make a cyclic peptide from the splicing intermediate.

Additionally, included in the invention is method for purifying a target molecule that binds a splicing intermediate from a mixture. This method includes the steps of: providing a solid phase support having the splicing intermediate specifically bound thereon; contacting the support with the target molecule in the mixture; washing the support to remove non-specifically bound matter from the support; and eluting the target molecule from the support.

As used herein, the phrase "non-naturally occurring" means being directly or indirectly made or caused to be made through human action. Thus, a non-naturally occurring nucleic acid molecule is one that has been produced through human manipulation, and not natural evolutionary processes.

By the phrase "nucleic acid molecule" is meant any chain of two or more nucleotides bonded in sequence. For example, a nucleic acid molecule can be a DNA or an RNA.

As used herein, the term "peptide" means a chain of two or more amino acids bonded in sequence, and includes polypeptides and proteins. By "polypeptide" is meant a polymer comprised of two or more peptides, regardless of length or post-translational modification. By "protein" is meant any chain of amino acids and includes peptides, polypeptides, proteins, and modified proteins such as glycoproteins, lipoproteins, phosphoproteins, metalloproteins, and the like.

A "linear peptide" is a peptide that is not in a circular form, and generally has both a

carboxy-terminal amino acid with a free carboxy-terminus and an amino-terminal amino acid with a free amino terminus.

In comparison, a "cyclic peptide" is a peptide that has been "cyclized." The term "cyclic" means having constituent atoms forming a ring. When referring to a peptide, the term "cyclize" means to make the peptide into a cyclic or "cyclized" form. Thus, for example, a linear peptide is "cyclized" when its free amino-terminus is covalently bonded to its free carboxy-terminus (i.e., in a head to tail format) such that no free carboxy- or amino-terminus remains in the peptide.

As used herein, a "splicing intermediate" is a polypeptide generated during the intein-mediated cyclization reaction illustrated above prior to the formation of the liberated cyclic peptide product. Splicing intermediates include "active-intein intermediates" (i.e., those with a chemical structure similar to the polypeptide labeled "A" in the above illustration), "thioester intermediates" (i.e., those with a chemical structure similar to the polypeptide labeled "B" in the above illustration), and "lariat intermediates" (i.e., those with a chemical structure similar to the polypeptide labeled "C" in the above illustration).

By the phrase "target peptide" is meant a peptide to be cyclized or displayed in a splicing intermediate. For example, a peptide interposed between a carboxy-terminal portion of a split intein and an amino-terminal portion of a split intein in a precursor protein would be a target peptide, if the peptide becomes cyclized upon splicing of the precursor protein or becomes a part of a splicing intermediate upon processing (e.g., folding) of the precursor protein.

As used herein, the word "intein" means a naturally-occurring or artificially-constructed polypeptide sequence embedded within a precursor protein that can catalyze a splicing reaction during post-translation processing of the protein. A list of known inteins is

published at <http://www.neb.com/inteins.html>. A "split intein" is an intein that has two or more separate components not fused to one another.

As used herein, the word "interposed" means placed in between. Thus, in a polypeptide having a first peptide interposed between a second and a third peptide, the chain of amino acids making up the first peptide is physically located in between the chain of amino acids making up the second peptide and the chain of amino acids making up the third peptide.

A plurality of peptides having "heterogenous amino acid sequences" means that the plurality of peptides is composed of at least two, but generally a large number of, different peptides of disparate amino acid sequence.

As used herein, the phrase "host system" refers to any medium or vehicle in which a nucleic acid molecule can be transcribed, replicated, and/or translated; and/or any medium or vehicle in which a polypeptide can be spliced or otherwise post-translationally processed.

As used herein, the word "spontaneously" means the action described occurs without the addition of an exogenous substance. For example, a precursor polypeptide within a host system spontaneously splices in the host system to yield a cyclic peptide when nothing is added to the host system other than the precursor polypeptide or a nucleic acid molecule encoding the precursor polypeptide. In comparison, a precursor polypeptide within a host system does not spontaneously splice in the host system if an agent extraneous to the host system is required to generate the cyclic peptide.

As used herein, the term "splice" or "splices" means to excise a central portion of the polypeptide to form two or more smaller polypeptide molecules. In some cases, splicing also includes the step of fusing together two or more of the smaller polypeptides to form a new polypeptide.

As used herein, the word "derived" means directly or indirectly obtained from, isolated from, purified from, descended from, or otherwise arising from.

As used herein, the phrase "expression vector" means a vehicle that facilitates transcription and/or translation of a nucleic acid molecule in a host system. An expression  
5 vector is "inducible" when adding an exogenous substance to a host system containing the expression vector causes the vector to be expressed (e.g., causes a nucleic acid molecule within the vector to be transcribed into mRNA).

By the phrase "expression of" a nucleic acid is meant that the nucleic acid is transcribed and/or translated into a polypeptide and/or replicated.

As used herein, the phrase "regulatory sequence" means a nucleotide sequence which modulates expression (e.g., transcription) of a nucleic acid molecule. For example, promoters and enhancers are regulatory sequences.

By the term "fused" is meant covalently bonded to. For example, a first peptide is fused to a second peptide when the two peptides are covalently bonded to each other (e.g., via  
15 a peptide bond).

As used herein an "isolated" or "substantially pure" substance is one that has been separated from components which naturally accompany it. Typically, a polypeptide is substantially pure when it is at least 50% (e.g., 60%, 70%, 80%, 90%, 95%, and 99%) by weight free from the other proteins and naturally-occurring organic molecules with which it is  
20 naturally associated.

A "progenitor DNA" is particular deoxyribonucleic acid from which mutations are made or based upon.

By the phrase "target molecule" is meant any molecule used to determine the binding or functional characteristics of another molecule.



Herein, "bind" or "binds" means that one molecule recognizes and adheres to another molecule in a sample, but does not substantially recognize or adhere to other molecules in the sample. One molecule "specifically binds" another molecule if it has a binding affinity greater than about  $10^5$  to  $10^6$  liters/mole for the other molecule.

5 A "cell-associated process" is one that takes place within a cell or in the near vicinity of the cell.

A "membrane-associated event" is a cell-associated process that takes place on the plasma membrane of a cell.

A "nuclear event" is a cell-associated process that takes place in the nucleus of a cell.

10 In comparison to a cell-associated event, an "extracellular reaction" is one that does not take place within a cell.

By the phrase "hybrid system" is meant two-hybrid systems, reverse two-hybrid systems, one-hybrid systems, split-hybrid systems, small molecule hybrid systems and all like systems for identifying interactions between peptides and other molecules (e.g., proteins and nucleic acid molecules). For a review of exemplary hybrid systems, see Vidal and Legrain,  
15 Nucleic Acids Res. 27:919, 1999.

Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Although methods and materials similar or equivalent to those described herein can be used  
20 in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions will control. In addition, the particular embodiments discussed below are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### Brief Description Of The Drawings

The invention is pointed out with particularity in the appended claims. The above and further advantages of this invention may be better understood by referring to the following description taken in conjunction with the accompanying drawings, in which:

Figure 1 is a schematic illustration of an overview of a general cyclization reaction within the invention.

Figure 2 is a schematic illustration of a series of chemical reaction steps that occur in a peptide cyclization method of the invention.

Figure 3 is a genetic map of (a) plasmid pARCP, (b) plasmid pARCP-DHFR, (c) plasmid pARCPAH-DHFR, (d) a modified vector having a cysteine (TGY) or serine (TCN) codon generated by cloning into the MfeI site (N represents any nucleobase, S represents C or G and Y represents pyrimidines), (e) plasmid pARCP-p, and (f) plasmid pARCBD-p.

Figure 4 is a photograph of a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of dihydrofolate reductase (DHFR) cyclization on a 10-20% gradient, Tris/glycine ready-gel (Biorad).

Figure 5 is a graph of DHFR activity of wild-type (triangles) and cyclic DHFR (diamonds) activity after preincubation at 65°C.

Figure 6 is a schematic illustration of the expected endoproteinase Lys-C digestion pattern for linear and cyclic DHFR.

Figure 7 is a photograph of FeCuY plates used in an *in vivo* assay to detect tyrosinase inhibition by pseudostellarin F.

Figure 8 is a schematic illustration of a method for purifying cyclic peptides within the invention.

Figure 9 is a schematic illustration of another method for purifying cyclic peptides within the invention.

5 Figure 10 is a schematic illustration of a solid phase support/affinity chromatography-based method for identifying/purifying molecules which specifically bind a splicing intermediate.

10 Figure 11 is a schematic illustration of another solid phase support/affinity chromatography-based method for identifying/purifying molecules which specifically bind a splicing intermediate.

Figure 12 is a schematic illustration of another solid phase support/affinity chromatography-based method for identifying/purifying molecules which specifically bind a splicing intermediate.

15 Figure 13 is a schematic illustration of another solid phase support/affinity chromatography-based method for identifying/purifying molecules which specifically bind a splicing intermediate.

Figure 14 is a schematic illustration of the use of aptamer scaffolds in the invention.

Figure 15 is a schematic illustration of two reactions for preparing aptamers within the invention.

20 Figure 16 is a schematic illustration of a method for screening within the invention.

Figure 17 is a schematic illustration of another method for screening within the invention

Figure 18 is a schematic illustration of another method for screening within the

invention.

Figure 19 is a schematic illustration of another method for screening within the invention.

#### Detailed Description

5           The trans-splicing ability of split inteins has been exploited to develop a general method of producing cyclic peptides and splicing intermediates displaying peptides in a looped conformation. In this method, a target peptide is interposed between two portions of a split intein in a precursor polypeptide. In an appropriate host system, the two portions of the split intein physically come together to form an active intein in a conformation that also forces the target peptide into a loop configuration. In this configuration, the ester isomer of the amino acid at the junction between one of the intein portions (e.g.,  $I_N$ ) and the target peptide is stabilized such that heteroatom from the other portion of the intein (e.g.,  $I_C$ ) can then react with the ester to form a cyclic ester intermediate. The active intein then catalyzes the formation of an aminosuccinimide that liberates a cyclized form of the target peptide (i.e., a lactone form), which then spontaneously rearranges to form the thermodynamically favored backbone cyclic peptide product (i.e., the lactam form). By arresting the reaction at given points before liberation of the cyclic peptide, splicing intermediates bearing the target peptide in a loop configuration can be produced. To produce such peptides, nucleic acid molecules encoding a polypeptide having the target peptide sequence interposed between the two intein portions can be constructed. Introduction of these constructs into an expression vector provides a method for producing the polypeptide in a host system, where the polypeptide can be spliced into a cyclic peptide or a splicing intermediate. Using this method, several different cyclic peptides or splicing intermediates can be prepared to generate a library of

cyclized or partially-cyclized peptides that can be screened for particular characteristics.

Referring to FIG. 1, an overview of an embodiment of the invention includes a method of making a cyclic peptide from a nucleic acid molecule. In this method, a nucleic acid molecule is prepared so that its nucleotide sequence encodes a polypeptide having in consecutive order a first portion of a split intein (e.g.,  $I_C$ ), a peptide to be cyclized (i.e., a target peptide), and a second portion of a split intein (e.g.,  $I_N$ ). The nucleic acid molecule can be incorporated into an expression vector to facilitate its expression in a host system where the nucleic acid can be transcribed and translated into a precursor polypeptide having the peptide to be cyclized interposed between the two split intein portions. By the steps described above, the two portions of the split intein come together and place the precursor peptide in a conformation that sets off chemical reactions that ultimately yield a cyclic peptide (see FIG. 2).

### Nucleic Acid Molecules

Nucleic acids molecules within the invention include those that encode a polypeptide having a first portion of a split intein, a second portion of a split intein, and a target peptide positioned in between the first portion of a split intein and the second portion of a split intein. In one embodiment of the invention, expression of the nucleic acid molecule in a host system results in a polypeptide that spontaneously splices in the host system to yield a cyclized form of the target peptide. In another embodiment of the invention, expression of the nucleic acid molecule in a host system results in a polypeptide that is a splicing intermediate of a cyclized form of the target peptide. The nucleic acids of the invention can be prepared according to the methods described herein, and can also be prepared using the guidance provided herein in conjunction with methods for preparing and manipulating nucleic acid molecules generally

known in the art (See, e.g., Ausubel et al. eds., Current Protocols in Molecular Biology, New York: John Wiley & Sons, 1997; Sambrook et al., Molecular Cloning: A laboratory Manual (2<sup>nd</sup> Edition), Cold Spring Harbor Press, 1989). For example, a nucleic acid molecule within the invention can be made by separately preparing a polynucleotide encoding the first portion of a split intein, a polynucleotide encoding the second portion of a split intein, and a polynucleotide encoding the target peptide. The three polynucleotides can be ligated together to form a nucleic acid molecule that encodes a polypeptide having the target peptide interposed between the first portion of a split intein and the second portion of a split intein.

#### Nucleic Acids Encoding Inteins

Nucleotide sequences that encode the first portion of a split intein and the second portion of a split intein of the nucleic acid molecules within the invention can be derived from known inteins. A fairly comprehensive and descriptive list of such inteins is published by New England Biolabs at [http://www.neb.com/inteins/int\\_reg.html](http://www.neb.com/inteins/int_reg.html). Any of these known inteins can be used as long as they are compatible with invention.

Nucleotide sequences that encode either naturally-occurring or artificially-produced split inteins can be used to generate the intein portions of nucleic acid molecules within the invention. Naturally-occurring split inteins are expressed in nature as two separate components that bind one another to form one active splicing agent. The nucleic acid molecules encoding these naturally-occurring components can thus be used in the invention. One example of a naturally-occurring split intein that may be used is Ssp DnaE (Wu et al, Proc. Natl. Acad. Sci. USA 95:9226,1998).

Inteins that are not split in their natural state (i.e., those that exist as one continuous chain of amino acids) can be artificially split using known techniques. For example, two or

more nucleic acid molecules encoding different portions of such inteins can be made so that their expression yields two or more artificially split intein components . See, e.g., Evans et al, J. Biol. Chem. 274:18359, 1999; Mills et al, Proc. Natl. Acad. Sci. USA 95:3543, 1998. The nucleic acids that encode such non-naturally occurring intein components (portions) can be used in the invention. Those nucleic acid molecules that encode non-naturally occurring split intein portions which efficiently interact on the same precursor polypeptide to yield cyclic peptides or splicing intermediates are preferred. Examples of non-naturally occurring split inteins from which such nucleic acid molecules can be derived include Psp Pol-1 (Southworth, M.W., et al, The EMBO J. 17:918, 1998), *Mycobacterium tuberculosis* RecA intein, (Lew, B.M., et al, J. Biol. Chem. 273:15887, 1998; Shingledecker, K., et al, Gene 207:187, 1998; Mills, K.V., et al, Proc. Natl. Acad. Sci. USA 95:3543, 1998), Ssp DnaB/Mxe GyrA (Evans, T.C. et al, J. Biol. Chem. 274:18359, 1999), and Pfu (Otomo et al, Biochemistry 38:16040, 1999; Yamazaki et al, J. Am. Chem. Soc.120:5591, 1998).

#### Nucleic acids encoding Target Peptides or Peptides Displayed in Splicing

##### Intermediates

Numerous methods of making nucleic acids encoding peptides of a known or random sequence are known in art. For example, polynucleotides having a predetermined or a random sequence can be prepared chemically by solid phase synthesis using commercially available equipment and reagents. Polymerase chain reaction can also be used to prepare polynucleotides of known or random sequences. See, e.g., Ausubel et al, *supra*. As another example, restriction endonucleases can be used to enzymatically digest a larger nucleic acid molecule or even whole chromosomal DNA into a plurality of smaller polynucleotide fragments that can be used to prepare nucleic acid molecules of the invention.

Polynucleotides that encode peptide sequences to be cyclized are preferably prepared so that one terminus of the polynucleotide encodes an asparagine, serine, cysteine, or threonine residue to facilitate the cyclization reaction. For the same reason polynucleotides that encode peptide sequences for production of splicing intermediates are preferably prepared so that the terminus encodes an amino acid other than an asparagine, serine, cysteine, or threonine residue so that the cyclization reaction is prevented.

#### Ligation Polynucleotides Encoding Intein Portions and Target Peptides or Peptides Displayed in Splicing Intermediates

Once generated, conventional methods can be used to ligate nucleic acid molecules encoding intein portions to a nucleic acid molecule encoding a target peptide (or peptide within a splicing intermediate) to form a larger nucleic acid molecule encoding a polypeptide having the first intein portion-target peptide-second intein portion order. See, e.g., Ausubel et al, *supra*.

#### Nucleic Acid Molecules that Encode Multiple Split Inteins and Multiple Peptides

Using techniques similar to those described above, one skilled in the art could also prepare nucleic acid constructs that encode more than one set of two portions of a split intein interposed with peptides. For example, the invention includes nucleic acids molecules encoding a precursor polypeptide molecules comprised of N polypeptides (N = an integer greater than or equal to 1) and having N target peptides interposed between 2N intein portions such that any target peptide i (i= an integer greater than 1 representing the position of an target peptide in the precursor polypeptide) is interposed between intein portion 2i-1 and 2i (e.g. target peptide 1 is between intein portion 1 & 2, target peptide 2 is between intein



portions 3 & 4 etc.). As long as intein portions 2i-1 and 2i are not complementary (i.e. able to physically interact to catalyze a splicing event), target peptide i can not cyclize. If, however, intein portion 2i is complementary with intein portion 2i+1 and intein portion 2N is complementary with intein portion 1, the entire ensemble of N polypeptides can perform N-1 trans splices (between 2 polypeptides) and 1 cis splice (ligating the two ends together) to give rise to a product wherein 1-N target peptides are covalently attached to one another in a cyclic peptide/protein (e.g., intein portions 2 & 3 trans-splice target peptides 1 & 2; intein portions four & five trans-splice target peptides 2 & 3; intein portions 2N-2 & 2N-1 trans-splice target peptides N-1 & N; and intein portions N & 1 cis-splice to close the cyclic product containing the N target sequences). The order of trans/cis splicing events is irrelevant. The slowest splicing species (whether it is the complementary intein portion 2N&1, 2&3 or 80&81) will by default perform the cis-splice.

Thus, nucleic acid constructs can be made that express two or more polypeptides each composed of a target peptide interposed between two portions of a split intein where the intein components are not complementary (i.e., do not derive from the same intein or otherwise cooperate to catalyze any of the cyclization reactions). In such constructs, no one polypeptide could be cyclized unless it was expressed in the presence of a second polypeptide having the appropriate complementary intein component. Constructs of such nucleic acids within the invention could encode only one polypeptide per construct or more than one polypeptide per construct (e.g., a bi-functional plasmid).

### Expression Vectors

The expression vectors of the present invention can be prepared by inserting polynucleotides encoding a target peptide into any suitable expression vector that can

facilitate expression of the polynucleotide in a host system. Such suitable vectors include plasmids, bacteriophages, and viral vectors. A large number of these are known in the art, and many are commercially available or obtainable from the scientific community. Those of skill in the art can select suitable vectors for use in a particular application based upon, e.g., the type of host system selected (e.g., in vitro systems, prokaryotic cells such as bacteria, and eukaryotic cells such as yeast or mammalian cells) and the expression conditions selected.

Expression vectors within the invention can include a stretch of nucleotides that encodes a target polypeptide and a stretch of nucleotides that operate as a regulatory domain that modulates or controls expression (e.g., transcription) of nucleotide sequences within the vector. For example, the regulatory domain can be a promoter or an enhancer.

Expression vectors within the invention can include nucleotide sequences that encode a peptide that facilitates screening of the cyclized form of the target peptide or splicing intermediate for a particular characteristic (e.g., an affinity tag such as a chitin-binding domain or a biotin tag; a colored or light-emitting label; a radioactive tag; etc. ), or purifying the cyclized form of the target peptide or splicing intermediate from a host system (e.g., an affinity tag such as a chitin-binding domain, a biotin tag, a colored or light-emitting label; a radioactive tag; etc. ).

In preferred embodiments, the expression vectors within the invention are produced with restriction sites both between and within the nucleic acid sequences that encode the split intein portions to enable the cloning of a wide variety of cyclization targets or splicing intermediates. In some embodiments, an expression vector of the invention can be an inducible expression vector, such as an arabinose inducible vector. Such vectors can be utilized to control expression of cyclization precursors or splicing intermediates within a host system. Other vectors can be selected for use in the invention based on their compatibility

with known bacterial expression strains and hybrid systems. See, e.g., Zhang et al, Curr. Biol. 9:417, 1999; Pellitier et al, Nat. Biotechnol. 17:683, 1999; Karimova et al, Proc. Natl. Acad. Sci. USA 95:5752, 1998; Dmitrova et al, Mol. Gen. Genet., 257:205, 1998; Xu et al, Proc. Natl. Acad. Sci. USA 96:151, 1999; Rossi et al, Proc. Natl. Acad. Sci. USA 94:8405, 1997.

### Polypeptides

Polypeptides within the invention include any that can be produced by expression of a nucleic acid of the invention. For example, a substantially pure precursor polypeptide that has a target peptide (or a peptide to be displayed by a splicing intermediate) interposed between the first portion of a split intein and the second portion of a split intein is included in the invention. In some embodiments of the precursor polypeptide, the target peptide may be directly fused to the first and second intein portions. The precursor polypeptide spontaneously splices in the host system to yield a cyclized form of the target peptide (or a splicing intermediate displaying a peptide).

Cyclized forms of target peptides and splicing inter-mediate displaying peptides are also within the invention. Preferably, these are produced by splicing of a precursor polypeptide of the invention. The cyclized forms of target peptides can be of any amino acid sequence that can be cyclized by the methods of the invention. The splicing intermediate can be an active intein intermediate, a thioester intermediate, or a lariat intermediate, and can display a peptide of any compatible amino acid sequence.

### Host Systems

Hosts systems that may be used in the invention include any systems that

support transcription, translation, and/or replication of a nucleic acid molecule of the invention; or that support post-translational modification (e.g., splicing) of a polypeptide or protein of the invention. Numerous such hosts systems are known. For example, in the invention, especially when it is desired to avoid artifacts or interference caused by living host systems, the host system can take the form of an in vitro transcription/translation system. Such systems can be fabricated in the laboratory according to published techniques or can be commercially purchased. For instance, STP2-T7 (cat. No. 69950-3) and STP-SP6 (cat. No. 69997-3) are available from Novagen (Madison, WI). Promega (Madison, WI) also sells such systems (e.g., cat. Nos. L1170, L2080, L4600, L4610, L4130, L4140, L1130, L1020, and L1030), as does Stratagene (La Jolla, CA) which markets a system branded IN VITRO EXPRESS (cat. No. 200360). Non-living host systems for use in the invention can also be derived from a living organism. For example, a cell lysate such as a reticulocyte lysate can be used in some applications.

Host systems can also take the form of living organisms. Living organisms are preferred for host systems because they can usually be reproduced in numerous copies thereby providing a continuous, readily-expandible, and easily-manipulated source of selected nucleic acid molecules. Living organisms that can be used as host systems within the invention include prokaryotes such as bacteria (e.g., *Escherichia coli*) and eukaryotes such as yeasts and mammalian (e.g., human, murine, bovine, ovine, porcine, etc.) cells. Archaeobacteria, plant cells, and any other organism suitable for use with the methods of the invention can also function as the host system.

The particular host system best suited for a particular application will vary depending on the many different factors. One of skill in the art, however, should be able to select a suitable host system for a particular application based on known applications of the different

host systems. For example, where large scale production of a cyclic peptide is desired, a bacterial host or an insect host would be suitable. As another example, where it is desired to analyze the interaction of human cell components, using a human cell as the host system would likely be more appropriate than using a bacterial system.

5 Method of Making a Polypeptide, Cyclic Peptide, or a Splicing Intermediate

10 The polypeptides of the invention can be prepared by conventional methods of producing polypeptides of a known amino acid sequence. For example, polypeptides within the invention can be made by solid phase synthesis using commercially available equipment and reagents. Known, in vitro methods of producing cyclic peptides can also be used to produce cyclic peptides. In many cases, however, the polypeptides of the invention are preferably produced by expressing nucleic acid molecules encoding them in a host system. For example, nucleic acid molecules within the invention can be incorporated into an expression vector and then introduced into a host system. The host system can then be placed under conditions that cause the vector to be expressed, resulting in the formation of a precursor peptide and subsequently a cyclized form of the target peptide or a splicing intermediate displaying the peptide.

15 A preferred method for making a cyclic peptide or a splicing intermediate includes the steps of: (a) providing an isolated nucleic acid molecule that encodes a polypeptide having a target peptide interposed between the first portion of a split intein and the second portion of a split intein; (b) providing a host system; (c) introducing the isolated nucleic acid molecule into the host system; and (d) expressing the isolated nucleic acid molecule. Expression of the nucleic acid molecule in the host system produces the peptide molecule in the form of a splicing intermediate of a cyclized form of the target peptide, or a polypeptide that

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spontaneously splices to yield a cyclized form of the target peptide.

In preferred embodiments of this method, production of the polypeptides, cyclic peptides, or splicing intermediates takes place in vivo (e.g., with a living host system) and in the absence of any exogenously-added agent, such as an agent to catalyze cyclization of a peptide (e.g., a protease or a thiol).

Production of polypeptides, cyclic peptides, or splicing intermediates can be monitored using standard techniques for characterizing proteins. See, e.g., Sambrook et al, *supra*. Exemplary techniques that can be used include conventional chromatography, HPLC, FPLC and the like; electrophoresis such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), 2-dimensional gel electrophoresis; electromagnetic radiation-based-spectroscopy, mass spectroscopy; analysis of enzymatic digestion products; thermostability assays; etc.

#### Purification of Polypeptides, Cyclic Peptides, or a Splicing Intermediates of the Invention

Conventional methods of purifying proteins can be adapted to purify the polypeptides, cyclic peptides, and splicing intermediates of the invention. The invention also includes a preferred method for purifying a cyclic peptide from a mixture. In this method, an affinity tag is attached to the cyclic peptide to aid in its purification. This method includes the steps of: (a) providing a mixture containing a cyclic peptide conjugated with an affinity tag; (b) mixing the conjugated cyclic peptide with a solid phase support having a ligand thereon that specifically binds the affinity tag so that the support becomes specifically bound with the cyclic peptide; (c) washing the support to remove non-specifically bound matter; and (d) eluting the cyclic peptide from the support.

In this method, the affinity tag can any molecule that can bind a ligand on a solid

phase support. For example, the affinity tag can be a chitin-binding domain where the ligand is chitin (see examples section below) or it can be a biotin tag where the ligand is streptavidin. Many other affinity tag-ligand pairs are known and can be used in the invention. Because the affinity tag specifically binds the ligand on the solid phase support, the cyclic peptides (with the attached affinity tag) will specifically bind the support. The support can then be washed with a buffer (e.g. a high salt, acid or alkaline buffer) that removes matter within the mixture that is non-specifically bound to the support. The affinity-tagged cyclic peptide can then be eluted from the solid phase support using a buffer containing a substance that separates the tag from the ligand (e.g., a competitive inhibitor such as excess unconjugated affinity tag; or a denaturing agent), or an enzyme or chemical reactant that cleaves the cyclic peptide from the affinity tag.

In an analogous manner, splicing intermediates rather than cyclic peptides can be purified. Cyclic peptides can also be purified from a mixture using splicing intermediates. For example, a method for purifying a cyclic peptide from a mixture includes the steps of: (a) providing a mixture containing a splicing intermediate conjugated with an affinity tag; (b) mixing the conjugated splicing intermediate with a solid phase support having a ligand thereon that specifically binds the affinity tag such that the support becomes specifically bound with the splicing intermediate; (c) washing the support to remove non-specifically bound matter; (d) adding to the support a reagent that makes a cyclic peptide from the splicing intermediate; and (e) eluting the cyclic peptide from the support. In a variation of the foregoing, steps (d) and (e) are reversed so that step (d) is eluting the splicing intermediate from the support and step (e) is adding to the eluted splicing intermediate a reagent that makes a cyclic peptide from the splicing intermediate. Reagents that may be added to make a cyclic peptide from a splicing intermediate include thiols, proteases, and other substances

which can catalyze cyclization of the splicing intermediate.

As a specific example, by fusing  $I_c$  to an affinity tag and removing the essential asparagine residue (see d in FIG. 3), a cyclic ester can be immobilized on an affinity column. The resulting cyclic peptide column can be used for the affinity purification of the cyclic peptide itself. A wide range of proteolytic methods can be employed to liberate the cyclic ester from the affinity tag and  $I_c$  depending upon the sequence of the cyclic peptide product.

Referring now to FIG. 8, a method for purifying cyclic peptides is shown. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) and to introduce an affinity tag downstream of  $I_N$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until the lariat intermediate is formed (step C). This molecule is then passed through an affinity column (step D) having a solid phase support with a ligand thereon that specifically binds the affinity tag and thus allows retention and purification of the  $I_N/I_C$  non-covalent complex (specie 3). The  $I_N/I_C$  reaction is then disrupted to yield a lariat intermediate (specie 4) which can be eluted from the affinity column. Proteolytic or chemical cleavage at amino acid Y (step F) liberates the lactone intermediate (specie 5). Acyl-to-N rearrangement (step G) yields the thermodynamically preferred amide cyclic product (specie 6).

Referring now to FIG. 9, a variation of the foregoing method for purifying cyclic peptides is shown. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) and to introduce an affinity tag upstream of  $I_C$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until a lariat intermediate is formed (step C). This molecule is then passed through an affinity column (step D) having a solid phase support with a ligand thereon that specifically binds the affinity tag/ $I_C$  intermediate (specie 3). Separation of the affinity tag



(step E) from the ligand (e.g., using a molecule that competitively inhibits the tag-ligand interaction, using a high salt buffer or denaturing agent, or using a chemical reagent or protease to cleave the tag) allows recovery of the lariat intermediate (specie 4). Proteolytic or chemical cleavage at amino acid Y (step F) liberates the lactone intermediate (specie 5).

5 Acyl-to-N rearrangement (step G) yields the thermodynamically preferred amide cyclic product (specie 6).

#### Method for Preparing a Library of Cyclic Peptides and Splicing Intermediates

Numerous methods of making linear peptide libraries are known in the art.

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10 Modifications of such known methods can be utilized with the methods of producing cyclic peptides and splicing intermediates taught herein to generate libraries of cyclic peptides and splicing intermediates. In general, a method of preparing a library of cyclic peptides and/or splicing intermediates includes the steps of: (a) providing a plurality of nucleic acid molecules encoding a plurality of target peptides having heterogenous amino acid sequences; (b) incorporating each of the plurality of nucleic acid molecules into an expression vector to form a plurality of expression vectors, whereby each of the plurality of nucleic acid molecules is interposed between a nucleic acid molecule encoding a first portion of a split intein and a nucleic acid molecule encoding an second portion of a split intein in each of the formed expression vectors, such that expression of the expression vectors in a host system results in the production of a plurality of splicing intermediates of cyclized forms of the target peptides or polypeptides that spontaneously splice in the host system to yield cyclized forms of the target peptides; and (c) expressing the expression vectors in the host system.

20 As more specific examples, the methods described in Childs et al, in Sequence Specificity in Transcription and Translation (Alan R. Liss, Inc., 1985) and the double strand

ligation method described in Schumacher et al, Science 271:1854, 1996 can be modified for use in the current invention. Known PCR-based methods can also be used to generate polynucleotides encoding peptides with random sequences that can be circularized or expressed as splicing intermediates in the invention. See, e.g., Caldwell and Joyce, PCR Methods Appl. 2:22, 1992; Ostermeier et al, Proc. Natl. Acad. Sci. USA 96:3562, 1999; the Nested Deletion Protocol and Reagents from Promega; and Stemmer, W.P. Nature 370:389, 1994 (DNA shuffling). The plurality of polynucleotides encoding peptides with heterogenous sequences can be incorporated as the target peptide (or the peptide to be displayed in a splicing intermediate) in the nucleic acid molecules and expression vectors of the invention as described above and then expressed in a host system to make a library of cyclic peptides or splicing intermediates.

#### Method for Screening a Cyclic Peptide for a Predetermined Characteristic

Myriad techniques exist for screening small molecules for particular characteristics. See, e.g., Fernades, P., Current Opin. Chem. Biol. 2:597, 1998; Science 286:1759, 1999; U.S. Patent Nos. 5,585,277 and 5,989,814. More specifically, methods for determining which peptide in a combinatorial peptide library binds specifically to a target protein are also known. E.g., U.S. Patent No. 5,834,318. Many of these methods can be adapted to screen cyclic peptides and/or splicing intermediates made with the methods of the invention for particular characteristics.

A general method of screening a peptide molecule for a predetermined characteristic includes the steps of: (a) providing a nucleic acid molecule that encodes a polypeptide having a target peptide interposed between a first portion of a split intein and a second portion of a split intein such that expression of the nucleic acid molecule in a host system produces the

peptide molecule either as a cyclized form of the target peptide (as a result of spontaneously splicing of the polypeptide in the host system) or a splicing intermediate of a cyclized form of the target peptide; (b) providing the host system; (c) introducing the isolated nucleic acid molecule in the host system; (d) placing the host system under conditions that cause the peptide molecule to be produced; and (e) testing the peptide molecule for the predetermined characteristic.

Step (a) can be performed as described elsewhere herein by, for example, using molecular biology techniques (see, Ausubel et al and Sambrook et al, *supra*) to produce polynucleotides encoding the target peptide, the first portion of an intein, and the second portion of an intein. The resulting three polynucleotides can then be fused (e.g., ligated) together to form the nucleic acid molecule. The host system provided can be any of those described herein in which the nucleic acid molecule can be expressed (e.g., a bacterium, a yeast, a mammalian cell, etc.). The nucleic acid can be introduced into the host system by known methods depending on the form of the nucleic acid molecule and the host system used. For example, the nucleic acid molecules can be introduced into a cell by electroporation, lipofection, using calcium chloride-mediated transformation, using a gene "gun," using a bacteriophage vector (when host system is a bacterium), using a plasmid construct, using a viral vector, etc.

The host system can be placed under conditions that cause the peptide molecule to be produced by adjusting the conditions according to the particular form of the nucleic acid molecule and the host system used. For a human cell host system, this can mean placing the cell in an appropriate nutrient rich medium and culturing the cell in a 37°C, humidified, 5-10% CO<sub>2</sub> incubator. For inducible expression vectors, this can mean adding the substance to the host system that induces expression of the nucleic acid molecule in the vector. For

example, when using an arabinose-inducible expression vector, this step can include adding arabinose to the host system.

Testing of the peptide molecule for the predetermined characteristic can be performed by a large number of different methods, e.g., measuring binding of the peptide molecule to a known ligand and analyzing the ability of the peptide molecule to modulate (i.e., increase or decrease the rate of) a biochemical reaction. For a description of various methods that may be used for testing peptides for predetermined characteristics see, Fernades, P., *supra*.

More specific exemplary methods that may be used to screen cyclic peptides or splicing intermediates for a particular characteristic include using a solid phase support and affinity chromatography to identify molecules which specifically bind cyclic peptides or splicing intermediates; using phage display technology; and using aptamer peptide fusion constructs and/or hybrid systems to identify cyclic peptides or splicing intermediates that can modulate a specific biochemical reaction or intracellular event.

#### Solid Phase Supports/Affinity Chromatography For Identifying Molecules That Interact with Cyclic Peptides and/or Splicing Intermediates

Cyclic peptides or splicing intermediates can be immobilized on a solid phase support to facilitate identification and/or purification of molecules that specifically bind a given cyclic peptide or splicing intermediate. For a general overview of peptide affinity columns for purification see Bumbach, G.A. and D.J. Hammond, *Biopharm.*, 5:24, 1992. Below are examples of how this can be performed in the invention.

Referring now to FIG. 10, a method for identifying/purifying molecules that specifically bind a given splicing intermediate is shown. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a

non-catalytic amino acid (Y) and to introduce an affinity tag upstream of  $I_C$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until a lariat intermediate is formed (step C). This molecule is then passed through an affinity column (step D) having a solid phase support with a ligand thereon that specifically binds the affinity tag/ $I_C$  intermediate (specie 3). A solution containing target molecules (i.e., candidates for binding the splicing intermediates) is then passed through the column (step E). Target molecules that specifically bind the splicing intermediate are selectively retained within the column. These target molecules can be removed from the column and biochemically analyzed (e.g., sequenced).

Referring now to FIG. 11, another method for identifying/purifying molecules which specifically bind a given splicing intermediate is shown. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) and to introduce an affinity tag downstream of  $I_N$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until a lariat intermediate is formed (step C). This molecule is then passed through an affinity column (step D) having a solid phase support with a ligand thereon that specifically binds the affinity tag to allow the retention and purification of the  $I_N/I_C$  non-covalent complex (specie 3). Cleavage of the affinity tag (step E) allows recovery of the lariat intermediate (specie 4). A solution containing target molecules (i.e., candidates for binding the splicing intermediates) is then passed through the column (step E). Target molecules that specifically bind the splicing intermediate are selectively retained within the column.

Referring now to FIG. 12, another method for identifying/purifying molecules that specifically bind a given splicing intermediate is shown. In this method, an active intein intermediate (specie 1) is mutagenized to replace the  $I_N$  nucleophile (step A) with a non-

1 catalytic amino acid (Z) and to introduce an affinity tag downstream of  $I_N$  (step B) to yield an  
2  $I_C$ -peptide- $I_N$ -tag fusion protein (specie 2). The intein-mediated cyclization reaction will  
3 produce the fusion protein (step C). This protein is then passed though an affinity column  
4 (step D) having a solid phase support with a ligand thereon that specifically binds the affinity  
5 tag to allow the retention and purification of the protein complex (specie 3). A solution  
6 containing target molecules (i.e., candidates for binding the splicing intermediates) is then  
7 passed through the column (step E). Target molecules that specifically bind the splicing  
8 intermediate are selectively retained within the column.

9 Referring now to FIG. 13, yet another method for identifying/purifying molecules  
10 which specifically bind a given splicing intermediate is shown. In this method, an active  
11 intein intermediate (specie 1) is mutagenized to replace the  $I_N$  nucleophile (step A) with a  
12 non-catalytic amino acid (Z) and to introduce an affinity tag upstream of  $I_C$  (step B) to yield  
13 an tag- $I_C$ -peptide- $I_N$  fusion protein (specie 2). The intein-mediated cyclization reaction will  
14 produce the fusion protein (step C). This protein is then passed though an affinity column  
15 (step D) having a solid phase support with a ligand thereon that specifically binds the affinity  
16 tag to allow the retention and purification of the protein complex (specie 3). A solution  
17 containing target molecules (i.e., candidates for binding the splicing intermediates) is then  
18 passed through the column (step E). Target molecules that specifically bind the splicing  
19 intermediate are selectively retained within the column.

## 20 Phage Display

21 Methods of screening molecules using phage display are also within the invention.  
22 Conventional methods using phage display can be modified by using the phage to display the  
23 cyclic peptides and/or splicing intermediates of the invention. For example, if Z in FIG. 1 is a

phage coat protein and  $XH=H$  in Fig. 2, the splicing reaction will not progress beyond the first ester intermediate, thus resulting in the target peptide being displayed as a loop. In this manner, libraries comprising phage particles displaying loop target peptides can be prepared and used to pan for molecules that bind the displayed loop. For instance, a target molecule can be immobilized on a solid phase support. Phage libraries displaying different looped peptides can then be mixed with the support. Those phage displaying looped peptides that bind the target molecule would be selectively retained on the support. After elution from the support (e.g., by cleavage of the ester linkage of the phage-displayed loop peptides with high concentrations of a potent nucleophile), the amino acid sequences of the looped peptides can be determined by standard molecular biology methods.

### Aptamers

Peptides aptamers are polypeptides that contain a conformationally constrained target peptide region of variable sequence displayed from a scaffold. Since cyclic peptides or splicing intermediates can function as aptamers, known methods of analyzing aptamers can be modified to assist in identifying particular characteristics of the cyclic peptides or splicing intermediates. See, e.g., Geyer et al, Proc. Natl. Acad. Sci. USA 96:8567, 1999; Caponigro et al, Proc. Natl. Acad. Sci. USA 95:7508, 1998; Mikhail et al, Proc. Natl. Acad. Sci. USA 95:14266, 1998; Norman et al, Science 285:591, 1999.

For example, referring to FIG. 14, cyclic proteins can be used as aptamer scaffolds in a technique that allows members of a peptide library to be displayed as a constrained loop between the N-terminus and C-terminus of the cyclic protein scaffold. As shown in FIG. 14, an aptamer library can be expressed as an  $I_C$ -scaffold- $I_N$  fusion protein (specie 1). Procession of the intein-mediated cyclization reaction in vivo (step A) yields an  $I_N$ , an  $I_C$ , and a cyclic

scaffold protein (specie 2). The aptamer library is displayed in the linker region between the N-terminus and C-terminus. In FIG. 14, N represents any amino acid and the subscripts  $n$  and  $m$  are any integral number equal or greater than 0, and X represents serine, threonine, or cysteine.

5 Other examples of using aptamers are also within the invention. Referring to FIG. 15, two such methods are described. In reaction I, an active intein intermediate is mutagenized to replace the nucleophilic amino acid from  $I_N$  (step A) with a non-catalytic amino acid (Z). These processes inactivate the splicing reaction to yield specie 2. Because of the strong interaction between  $I_C$  and  $I_N$ , this technique allows the members of a peptide library to be  
10 displayed as a constrained loop in the linker region between the two intein portions (Target). In reaction II, an active intein intermediate is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) to yield specie 2. Progression of the intein-mediated cyclization reaction proceeds in vivo (step B) and arrests at the lariat intermediate stage (specie 3), allowing members of a peptide library to be displayed as a constrained  
15 lactone.

### Hybrid Systems

The yeast two-hybrid system is a well-studied method for analyzing in vivo protein-protein interactions. Fields, S. and O. Song, Nature (London) 340:245, 1989. It and variations thereof such as one-hybrid systems, three-hybrid systems, reverse two-hybrid  
20 system, split-hybrid system, alternative n-hybrid systems, small molecule-based hybrid systems can be used to analyze the characteristics of cyclic peptides and/or splicing intermediates by adapting known methods. See, e.g., Drees, B. L., Current Opin. Chem. Biol., 3:64, 1999; Vidal, M., and P. Legrain, Nucleic Acids Research, 27:919, 1999; Current



Protocols in Molecular Biology, eds., Ausubel, F. M., et al, Wiley, New York, 1996; Huang, J. and S.L. Schreiber, Proc. Natl. Acad. Sci. (USA) 94:13396, 1997; Yang, M., et al, Nucleic Acids Res. 23:1152, 1995; Colas, P., et al, Nature (London), 380:548, 1996; Xu, C.W., et al, Proc. Natl. Acad. Sci. (USA) 94:12473, 1997.

5 For example, referring to FIG. 16, a method of identifying a target protein that interacts with a splicing intermediate is within the invention. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) and to introduce a DNA-binding domain downstream of  $I_N$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until the lariat intermediate (specie 3) is formed (step C).  $I_N$  and  $I_C$  form a strong non-covalent complex. The resulting lariat intermediate is then co-expressed with a target protein attached to a DNA-binding domain (step D). Interaction of the lariat intermediate with the target protein (specie 4) causes activation of a promoter region (step E) leading to expression of the reporter gene (\*). This method allows identification of target molecules able to bind the lariat intermediate. This method can be modified such that a known molecule (in place of an unknown target protein) is attached to a DNA-binding domain, so that lariat intermediates displaying a looped peptide that binds the known molecule can be identified.

Referring to FIG. 17, another method for identifying a target protein that interacts with a splicing intermediate is described. In this method, an active intein intermediate (specie 1) is mutagenized to replace the catalytic asparagine (step A) with a non-catalytic amino acid (Y) and to introduce a DNA-binding domain upstream of  $I_C$  (step B) to yield specie 2. The intein-mediated cyclization reaction will proceed until the lariat intermediate (specie 3) is formed (step C). This molecule is then co-expressed with a target protein attached to a DNA-binding domain (step D). Interaction of the lariat intermediate with the target protein (specie

4) causes activation of a promoter region (step E) leading to expression of the reporter gene (\*). This method allows identification of target molecules able to bind the lariat intermediate. This method can be modified such that a known molecule (in place of an unknown target protein) is attached to a DNA-binding domain, so that lariat intermediates displaying a looped peptide that binds the known molecule can be identified.

Referring now to FIG. 18, another method for identifying a target protein that interacts with a splicing intermediate is described. In this method, an active intein intermediate (specie 1) is mutagenized to replace the  $I_N$  nucleophile (step A) with a non-catalytic amino acid (Z) and to introduce a DNA-binding domain (DBD) downstream of  $I_N$  (step B). These processes will inactivate the splicing reaction and will generate an  $I_C$ -peptide- $I_N$ -DBD fusion protein (specie 2). This molecule is then co-expressed with a target protein attached to a DNA-binding domain (step C). Interaction of the fusion protein with the target protein (specie 3) causes activation of a promoter region (step D) leading to expression of the reporter gene (\*). This method allows identification of target molecules able to bind the fusion protein. This method can be modified such that a known molecule (in place of an unknown target protein) is attached to a DNA-binding domain, so that fusion proteins displaying a looped peptide that binds the known molecule can be identified.

Referring to FIG. 19, yet another method for identifying a target protein that interacts with a splicing intermediate is described. In this method, an active intein intermediate (specie 1) is mutagenized to replace the  $I_N$  nucleophile (step A) with a non-catalytic amino acid (Z) and to introduce a DNA-binding domain upstream of  $I_C$  (step B). These processes will inactivate the splicing reaction and will generate a DBD- $I_C$ -peptide- $I_N$  fusion protein (specie 2). This molecule is then co-expressed with a target protein attached to a DNA-binding domain (step C). Interaction of the fusion protein with the target protein (specie 3) in step D

causes activation of a promoter region (step E) leading to expression of the reporter gene (\*). This method allows identification of target molecules able to bind the fusion protein . This method can be modified such that a known molecule (in place of an unknown target protein) is attached to a DNA-binding domain, so that fusion proteins displaying a looped peptide that binds the known molecule can be identified.

#### Targeting Cyclic Peptides and Splicing Intermediates In Vivo

The cyclic peptides and splicing intermediates within the invention can be specifically targeted to particular cellular locales or for extracellular secretion by using modifications of targeting methods known in the art . See, e.g., Wilkinson et al, J. Membrane Biol., 155:189, 1997; Komiya et al, The EMBO J., 17:3886, 1998; Kouranov and Schnell, J. Biol. Chem. 271:31009, 1996; Bhagwat et al, J. Biol. Chem., 274:24014, 1999; Adam, S.A., Current Opin. Cell Biol. 11:402-406, 1999; Gorlich, D., Current Opin. Cell Biol. 9:412, 1997; Pemberton et al, Current Opin. Cell Biol. 10:292, 1998; Sakaguchi, M., Current Opin. Cell Biol. 8:595, 1997; Folsch et al., The EMBO J. 17:6508, 1998. For example, various signal peptides can be attached to the cyclic peptides or splicing intermediates of the invention to cause them to localize to predetermined cellular compartments or to be secreted into the extracellular space after translation. In this manner, the cyclic peptides or splicing intermediates of the invention can be targeted to cellular locales such as mitochondria, lysosomes, endoplasmic reticula, chloroplasts, golgi, periplasm, the nucleus, the plasma membrane. This method for targeting can also be used in the methods for generating a peptide libraries and methods of screening such libraries where it is desired to identify molecules that interact or exhibit an activity at predetermined cellular locations.

## Examples

### Preparation of Cyclic Dihydrofolate Reductase (DHFR) and Cyclic Pseudostellarin F

#### Materials and Methods

#### Vector Construction

5       The gene for the Ssp DnaE N-intein ( $I_N$ ) was amplified from Ssp 6803 genomic DNA with *Taq* polymerase and primers introducing 5'-*Bgl*II and *Nsi*I and 3'-*Pst*I restriction sites. The Ssp DnaE  $I_C$  gene was amplified similarly with primers introducing 5'-*Nco*I and 3'-*Nde*I and *Sac*I restriction sites. Plasmid pDIMCP resulted from individually cloning the intein fragments into pDIMC7 [identical to pDIMC6 (see Ostermeier et al., Proc. Natl. Acad. Sci. USA 96:3562, 1999) except for conversion of a *Bam*HI restriction site into *Bgl*II]. An alanine to histidine mutation in the  $I_C$  gene (A35H) was affected by Quick-Change mutagenesis (Stratagene) resulting in pDIMCPAH. Excision of the intein fragments as an *Nco*I/*Pst*I digest and ligation into pAR4 [derived from pAR3 (Perez-Perez, J. and J. Gutierrez, Gene, 158:141, 1995; American Type Culture Collection (ATCC) #87026) with a unique *Nco*I in the multiple cloning site] generated pARCP (a in Fig. 3) and pARCPAH. *E. coli* DHFR was amplified from pET22b-DHFR (Miller, G.P. and S.J. Benkovic, Biochemistry 37:6327, 1998) with primers introducing a 5'-*Nde*I site followed by (CAC)<sub>6</sub> (encoding six histidine residues) and a 3'-*Pst*I site, digested with *Nde*I/*Pst*I and ligated into *Nde*I/*Nsi*I digested pARCP or pARCPAH to produce pARCP-DHFR (b in Fig. 3) and pARCPAH-DHFR (c in Fig. 3). A polyhistidine sequence was prepared synthetically with *Nde*I, *Nsi*I and *Bsp*MI sites, and ligated into pARCPAH to produce plasmid pARCP2-6H which encodes cyclo-[CHMHHHHHHGAGAA]. Plasmid pARCP-p was produced in three steps from pDIMCPAH: (1) Quick-Change mutagenesis introduced an *Afl*II site into  $I_N$ , generating pDIMCPMA; (2) the pseudostellarin F gene was synthetically prepared and ligated into

*MfeI/AflI* digested pDIMCPMA to produce pDIMCP-p; and (3) the fusion construct was excised from pDIMCP-p as an *NcoI/PstI* fragment and ligated into *NcoI/PstI* digested pAR4 to produce pARCP-p (e in Fig. 3). To produce plasmid pARCBD-p, a *KpnI* site was introduced at the carboxyl terminus of the  $I_N$  gene of pARCP-p by Quick-Change mutagenesis to produce pARCPpK. The gene encoding the chitin binding domain was amplified from plasmid pCYBI (New England Biolabs, Inc., Beverly, Massachusetts) with primers introducing a 5' *KpnI* site and a 3' *HindIII* site. Both the PCR product and pARCPpK were digested with *KpnI* and *HindIII* and ligated together to generate pARCBD-p (f in Fig. 3). All enzymes were from Promega or New England Biolabs unless otherwise noted.

#### DHFR purification

XL I -Blue cells harboring either pARCP-DHFR or pARCPAH-DHFR were grown in LB medium plus 50 ug/ml chloramphenicol at 37°C until the culture reached an OD<sub>600</sub> Of 0.7. The culture was induced with L-(+)-arabinose to a final concentration of 0.5% and grown at 28°C for 24 hours. Cells were harvested by centrifugation (7,000 x g, 10 minutes) and frozen in liquid nitrogen. The cells were lysed, and DHFR containing proteins were purified as described (Miller and Benkovic, *id*). The cyclic product was separated from other DHFR-containing intermediates by FPLC using a Mono-Q column (Amersham Pharmacia) eluted with a gradient of 0 -1 M NaCl in 50 mM Tris-HCl over 30 minutes. Western blotting was performed with anti-His (Qiagen) and goat anti-mouse-alkaline phosphatase-conjugated antibodies (Pierce) according to the manufacturers' instructions.

#### Endoproteinase Lys-C Digestion

Wild-type or cyclic DHFR (50 ug) was treated with 0.5 ug of endoproteinase Lys-C in 0.1 M  $\text{NH}_4\text{HCO}_3$  at 37°C. Samples were taken at 6 and 24 hours, visualized on a SDS/16% PAGE gel and submitted for matrix assisted, laser desorption ionization (MALDI) time-of-flight mass spectrometry (Moore, W. T., Methods Enzymol., 289:520, 1997).

#### DHFR assays

Thermostability was assayed by preincubation of 100 nM wild type or cyclic DHFR at either 25°C or 65°C in MTEN buffer [50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine and 100 mM NaCl]. Aliquots were taken at various time points and equilibrated to room temperature for five minutes in the presence of 100 uM 7, 8-dihydrofolate. Activity assays were initiated with reduced nicotinamide adenine dinucleotide (NADPH) as previously described (Miller and Benkovic, *supra*).

#### Synthesis of cyclo-[Ser-Gly-Gly-Tyr-Leu-Pro-Pro-Leu]

To a solution of 3.5 mg (4 umol) of  $\text{NH}_2\text{-Ser-Gly-Gly-Tyr-Leu-Pro-Pro-Leu-CO}_2\text{H}$  and 1.8 mg (16 umol) of *N*-hydroxysuccinimide in 20 ml of dimethylformamide was added 3.0 mg (16 umol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The reaction was stirred for 10 hours at 25°C. An additional 3.0 mg of EDC was then added and stirring was continued at 25°C for another 10 hours. The solvent was removed by rotary evaporation and the residue was dissolved in 2 ml of water for purification by reversed-phase HPLC on a Whatman Partisil 10 ODS-3 9.4-mm X 50-cm column eluted with a linear gradient of 0-50% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid/water over 30 minutes. The appropriate fractions were lyophilized to yield 2.8 mg (80%) of a white solid,  $[\text{m/z } 785 (\text{MH}^+)]$ .  $^1\text{H-NMR}$

and UV-visible spectra of the synthetically prepared material were consistent with published spectra for the isolated natural product (Morita, H., et al, Tetrahedron 50:9975, 1994).

#### Pseudostellarin F Purification

*E. coli* strains XL1-Blue, DH5a or BL21-DE3 harboring pARCP-p were grown and harvested as described for DHFR purification. The media (500 ml) was extracted three times with 1-butanol (3 x 100 ml). The extracts were combined and evaporated, and the solid residue was resuspended in 2 ml 0.1 M K<sub>2</sub>HP0<sub>4</sub> (pH 8.0; lysis buffer). Cells were resuspended in 10 ml of lysis buffer, sonicated, and clarified by centrifugation (20,000 x g, 20 minutes). The lysate was extracted (3 x 5 ml of *n*-butanol), and extracts were combined, evaporated and resuspended in 500 ul of lysis buffer. The recombinant product was purified from lysate and media extracts by HPLC as described above. Lyophilization of the appropriate fractions from the lysate and media extractions yielded an oily residue, *m/z* 785.47 (MH<sup>+</sup>), 807.43 (MNa<sup>+</sup>) and 823.44 (MK<sup>+</sup>). <sup>1</sup>H-NMR and UV-visible spectra of the recombinant material were consistent with published spectra for the isolated natural product (Morita et al, *supra*). Proteins fused to the chitin binding domain were prepared as described above through generation of the clarified lysate. The lysate was passed over a chitin column (New England Biolabs, Inc.) equilibrated with lysis buffer. The column was eluted isocratically, and fractions containing splicing intermediates were pooled and submitted for MALDI mass spectral analysis.

#### Tyrosinase Cloning

The tyrosinase gene (including ORF 438) from *Streptomyces antibioticus* (Bernan et al, Gene 37:101, 1985) was amplified with Vent -polymerase from pIJ702 (ATCC no. 35287)

with primers introducing 5' *Nde*I and 3' *Eco*RI restriction sites. The PCR product was digested with *Nde*I and *Eco*RI and ligated into similarly digested pDIMN2 (Ostermeier et al, *supra*) to generate pDIMN-Y. Transformed ligation mixtures were grown at ambient temperature for 5 days, and colonies that expressed tyrosinase were identified by pigment formation on FeCuY plates [LB agar plates containing ampicillin (200 ug/ml),  $FeCl_3 \cdot 6H_2O$  (0.2 mM),  $CuSO_4 \cdot 5H_2O$  (0.2 mM), L-tyrosine (0.3 mg/ml, *Y*) and isopropyl-B-D-galactoside (1 mM)] (Della-Cioppa, G. et al., Biotechnology 8:634, 1990).

## Results

### Design of Genetic Constructs

The genes encoding Ssp  $I_C$  and  $I_N$  were amplified from Ssp genomic DNA by standard molecular biology methods (Sambrook, et al, *supra*) and serially ligated into pDIMC7. The resulting cyclization precursor (CP) fragment was excised from pDIMC7 and cloned adjacent to the AraB promoter of pAR3 to generate the pARCP vector series (Fig. 3). These vectors activate the expression of cyclization precursors in the presence of arabinose. The *E. coli* DHFR gene was cloned between the *Nde*I and *Nsi*I sites of pARCP to create an in frame fusion with each of the split intein genes (b and c in Fig. 3). The PCR primer used to amplify DHFR also introduced a sequence encoding a six-histidine tag at the 5' end of the DHFR gene to allow immunodetection of the region to be cyclized. Two DHFR constructs were assembled in order to investigate the role of the penultimate residue of  $I_C$  in acid/base catalysis of asparagine side chain cyclization. Plasmid pARCP-DHFR (b in Fig. 3) encodes wild type  $I_C$ , which has an alanine residue neighboring the terminal asparagine. Plasmid pARCPAH-DHFR (c in Fig 3) incorporates an alanine-to-histidine mutation at the penultimate position in  $I_C$  gene. To produce pseudostellarin F (cyclo-[SGGYLPPL]), the



vector was modified by silent mutation to create an *Afl*III site at the 5'-end of the  $I_N$  gene (d in Fig. 3). An *Mfe*I site occurs naturally at the 3'-end of the wild-type  $I_C$  gene. Ligation of a synthetically prepared, double-stranded insert encoding pseudostellarin F into the modified vector produced plasmid pARCP-p (e in Fig. 3). A *Kpn*I site was introduced at the 3'-end of the  $I_N$  gene in order to fuse the gene for the chitin-binding domain to the pseudostellarin-producing construct (f in Fig. 3).

#### Production and Characterization of Cyclic DHFR

DHFR cyclization was readily apparent by SDS-PAGE upon arabinose induction of pARCP-DHFR as shown in FIG. 4 (F:  $I_C$ -DHFR- $I_N$  fusion protein. T:  $I_C$ -DHFR- $I_N$  fusion thioester intermediate. R:  $I_C$ -DHFR lariat intermediate. L: linear DHFR. 0: cyclic DHFR.  $I_N$ : N-intein.  $I_C$ : C-intein. Lane 1: uninduced XLI-Blue/pARCP-DHFR. Lane 2: arabinose induced XLI-Blue/pARCP-DHFR. Lane 3: arabinose induced XLIBLue/pARCPAH-DHFR. Lane 4: lane 3 crude lysate after methotrexate agarose. Lane 5: lane 4 material post FPLC. Lane 6: Wild-type DHFR).

Bands with apparent molecular weights corresponding to the linear (L, 23 kDa) and cyclic (0, 21 kDa) DHFR products, the fusion protein (F, 37 kDa),  $I_N$  (14 kDa) and  $I_C$  (4 kDa) were clearly visible, as were bands tentatively assigned as the thioester (T, 36 kDa) and lariat intermediates (R, 26 kDa) (Fig. 4, lane 2). Mutation of the penultimate residue of  $I_C$  (A35) from alanine to histidine (c in Fig. 3) improved the yield of cyclic DHFR (Fig. 4, lane 3). Methotrexate-agarose affinity chromatography of the crude lysate (Fig. 4, lane 4) confirmed that the majority of the induced bands contained correctly folded DHFR. Although  $I_N$  is not covalently attached to DHFR, it was retained on the methotrexate column presumably due to non-covalent complex formation with the  $I_C$ -DHFR lariat intermediate (R). The

methotrexate-agarose eluant was fractionated by FPLC, allowing purification of 5 mg of the cyclic product per liter of culture (Fig. 4, lane 5). Western blotting (not shown) with an anti-His antibody demonstrated the presence of the polyhistidine linker sequence (d in Fig. 3) in the FPLC-purified protein. The protein migrated more rapidly in SDS/PAGE analyses than recombinant DHFR (Fig. 4, lane 6) despite the extra 11-amino acid linker sequence (b in Fig. 3) implying an additional topological constraint. Furthermore, no reaction was detected when the FPLC-purified protein was reacted with phenylisothiocyanate (Edman, P., Acta Chem. Scand., 4:283, 1950), suggesting that the amino terminus was unavailable.

Cyclic DHFR had steady-state kinetic parameters and substrate, cofactor and methotrexate dissociation constants which were indistinguishable from the wild type enzyme at 25°C. Activity assays conducted after 65°C preincubation of wild type and cyclic DHFR indicated that cyclization improved the thermostability of the enzyme (Fig. 5).

Endoproteinase Lys-C digestion was used to demonstrate unambiguously that the FPLC purified protein was cyclic DHFR. Digestion of the wild type enzyme produces amino-terminal (4.4 kDa) and carboxy-terminal (6.3 kDa) fragments; in a cyclic protein, these two fragments would be joined, resulting in a 10.7 kDa digestion product (Fig. 6). The FPLC purified material was resistant to proteolysis compared to the wild type enzyme, and mass spectral analysis of the digestion mixtures identified a 10.7 kDa peak in the product resulting from the cyclic protein, which was absent in the wild type enzyme (data not shown).

#### Production and Characterization of Pseudostellarin F

Pseudostellarin F production was readily detected *in vivo* through inhibition of recombinant *Streptomyces antibioticus* tyrosinase (FIG. 7). In the experiment shown in FIG. 7, XLI-Blue cells were co-transformed with pDIM-NY and either pARCP2-6H (a & b) or

pARCP-p (c & d). The cells were plated on FeCuY plates with chloramphenicol (50 ug/ml), either without (a & c) or with (b & d) L-(+)arabinose (0.5%).

Co-expression of pseudostellarin F in tyrosinase expressing cells dramatically reduced pigment formation (d in Fig. 7). Expression of an unrelated cyclic peptide from pARCP2-6H failed to inhibit tyrosinase (a and b in Fig. 7), and inhibition absolutely required arabinose induction (compare c and d in Fig. 7). SDS/PAGE analysis of arabinose-induced pARCP-p in several bacterial strains (BL21-DE3, DH5a, and XLI-Blue) allowed the visualization of bands corresponding to the fusion protein (F), thioester intermediate (T) and  $I_N$ . An intense, low molecular weight band was also visible, but the resolution was insufficient to separate the lariat intermediate (R) and  $I_C$  (data not shown). Although pseudostellarin F was too small to be visualized by SDS/PAGE, mass spectral analysis indicated its presence in both the crude cell lysate and media. Approximately 30 ug of the recombinant cyclic peptide was isolated from the cell lysate per gram of wet cell mass. Pseudostellarin F was also isolated from the media by 1-butanol extraction followed by HPLC with a yield that varied between 2 mg/liter (XLI Blue) and 20 mg/liter (BL21-DE3) depending on the expression strain. The NMR spectrum of the recombinant material was consistent with that reported for the natural product (Morita et al., *supra*), and the retention time of the bacterially expressed cyclic peptide was identical to a synthetically prepared standard. The recombinant material failed to react with ninhydrin (see, Gordon, A.J., and R.A. Ford, The Chemist's Companion: A Handbook of Practical Data, Techniques, and References, Wiley Interscience, New York, 1972), indicating a backbone cyclic peptide (lactam) rather than a lactone product. Neither HPLC nor mass spectral analysis provided any evidence for production of the linear parent peptide.

A chitin-binding domain was fused to the carboxy-terminus of  $I_N$  to affinity-purify intermediates of the intein-mediated ligation reaction and characterize them by MALDI mass

spectrometry (Table 1).

Reaction Component	Mass, Da		
	Linear	Cyclic	Observed
F, T	24,380.5	NA	24,380.4
I <sub>N</sub>	19,642.0	NA	19,642.3
R	4,756.5	4,738.5	4,756.2
I <sub>c</sub>	3,969.2	3,951.2	3,953.0
Pseudostellarin F	802.4	784.4	784.4

Table 1: Mass spectral characterization of pseudostellarin F cyclization intermediates

All of the intermediates of the splicing reaction, including I<sub>c</sub>, were retained when the crude cell lysate from arabinose-induced pARCBd-p in XL1-Blue was passed over a chitin affinity column. Pseudostellarin F was recovered from the unretained material by 1-butanol extraction. The observed molecular masses for the fusion protein (F), the thioester intermediate (T) and I<sub>N</sub> were in excellent agreement with the values predicted from the gene sequence. The mass of I<sub>c</sub> was consistent with the asparagine-cyclized form as predicted from the proposed mechanism of product release. The molecular mass of the lariat intermediate (R) was more consistent with the linear I<sub>c</sub>-pseudostellarin F fusion product than the branched lactone product expected from the transesterification reaction.

#### Other Embodiments

While the above specification contains many specifics, these should not be construed as limitations on the scope of the invention, but rather as examples of preferred embodiments

thereof. Many other variations are possible. Accordingly, the scope of the invention should be determined not by the embodiments illustrated, but by the appended claims and their legal equivalents.

What is claimed is:

1           1.     A non-naturally occurring nucleic acid molecule encoding a polypeptide  
2 comprising a first portion of a split intein, a second portion of a split intein, and a target  
3 peptide interposed between the first portion of a split intein and the second portion of a split  
4 intein;

5           wherein expression of the nucleic acid molecule in a host system produces the  
6 polypeptide in a form selected from the group consisting of: (a) a polypeptide that  
7 spontaneously splices in the host system to yield a cyclized form of the target peptide, and (b)  
8 a splicing intermediate of a cyclized form of the target peptide.

1           2.     The non-naturally occurring nucleic acid molecule of claim 1, wherein the  
2 polypeptide is a polypeptide that spontaneously splices in the host system to yield a cyclized  
3 form of the target peptide.

1           3.     The non-naturally occurring nucleic acid molecule of claim 1, wherein the  
2 polypeptide is a splicing intermediate of a cyclized form of the target peptide.

1           4.     The non-naturally occurring nucleic acid molecule of claim 1, wherein  
2 both the first portion of a split intein and the second portion of a split intein are derived from  
3 a naturally-occurring split intein.

1           5.     The non-naturally occurring nucleic acid molecule of claim 4, wherein  
2 both the first portion of a split intein and the second portion of a split intein are derived from

3 Ssp DnaE.

1 6. The non-naturally occurring nucleic acid molecule of claim 1, wherein  
2 at least one of the first portion of a split intein and the second portion of a split intein is  
3 derived from a non-naturally occurring split intein.

1 7. The non-naturally occurring nucleic acid molecule of claim 6, wherein the  
2 non-naturally occurring split intein is derived from the group consisting of RecA, DnaB, Psp  
3 Pol-I, and Pfu inteins.

1 8. The non-naturally occurring nucleic acid molecule of claim 1, wherein  
2 both the first portion of a split intein and the second portion of a split intein are derived from  
3 a non-naturally occurring split intein.

1 9. The non-naturally occurring nucleic acid molecule of claim 3, wherein the  
2 splicing intermediate is an active intein intermediate.

1 10. The non-naturally occurring nucleic acid molecule of claim 3, wherein the  
2 splicing intermediate is a thioester intermediate.

1 11. The non-naturally occurring nucleic acid molecule of claim 3, wherein the  
2 splicing intermediate is a lariat intermediate.

1 12. A non-naturally occurring nucleic acid molecule encoding a polypeptide

comprising a first portion of a split intein, a second portion of a split intein, a third portion of a split intein, and fourth portion of a split intein, wherein a first target peptide is interposed between the first portion of a split intein and the second portion of a split intein, and a second target peptide is interposed between the third portion of a split intein and the fourth portion of a split intein.

13. The non-naturally occurring nucleic acid molecule of claim 12 wherein the first portion of a split intein is complementary to the third portion of a split intein but not complementary to the second portion of a split intein, and the second portion of a split intein is complementary to the fourth portion of a split intein but not complementary to the third portion of a split intein.

14. An expression vector comprising a nucleic acid molecule that encodes a polypeptide comprising a first portion of a split intein, a second portion of a split intein, and a target peptide interposed between the first portion of a split intein and the second portion of a split intein, wherein expression of the nucleic acid molecule in a host system produces the polypeptide in a form selected from the group consisting of: (a) a polypeptide that spontaneously splices in the host system to yield a cyclized form of the target peptide, and (b) a splicing intermediate of a cyclized form of the target peptide.

15. The expression vector of claim 14, wherein the polypeptide is a polypeptide that spontaneously splices in the host system to yield a cyclized form of the target peptide.

16. The expression vector of claim 14, wherein the polypeptide is a splicing



2 intermediate of a cyclized form of the target peptide.

1 17. The expression vector of claim 15, wherein the nucleic acid molecule further  
2 comprises a regulatory sequence that facilitates expression of the polypeptide in the host  
3 system.

1 18. The expression vector of claim 14, wherein the nucleic acid molecule  
2 further comprises a nucleotide sequence that encodes a peptide that facilitates screening of the  
3 cyclized form of the target peptide for a particular characteristic.

1 19. The expression vector of claim 14, wherein the nucleic acid molecule further  
2 comprises a nucleotide sequence that encodes a peptide that facilitates purifying the cyclized  
3 form of the target peptide from the host system.

1 20. The expression vector of claim 14, wherein the target peptide has a first end  
2 fused to the first portion of a split intein and a second end fused to the second portion of a  
3 split intein.

1 21. The expression vector of claim 14, wherein both the first portion of a split  
2 intein and the second portion of a split intein are derived from a naturally-occurring split  
intein.

1 22. The expression vector of claim 21, wherein both the first portion of a split  
2 intein and the second portion of a split intein are derived from Ssp DnaE.

1           23.    The expression vector of claim 14, wherein at least one of the first portion of a  
2 split intein and the second portion of a split intein is derived from a non-naturally occurring  
3 split intein.

1           24.    The expression vector of claim 23, wherein the non-naturally occurring split  
2 intein is derived from the group consisting of RecA, DnaB, Psp Pol-I, and Pfu inteins.

1           25.    The expression vector of claim 14, wherein both the first portion of a split  
2 intein and the second portion of a split intein are derived from a non-naturally occurring split  
3 intein.

1           26.    The expression vector of claim 16, wherein the splicing intermediate is a  
2 active intein intermediate.

1           27.    The expression vector of claim 16, wherein the splicing intermediate is a  
2 thioester intermediate.

1           28.    The expression vector of claim 16, wherein the splicing intermediate is a lariat  
2 intermediate.

1           29.    The expression vector of claim 14, wherein the host system comprises a  
2 prokaryotic cell.

1 30. The expression vector of claim 29, wherein the prokaryotic cell is a bacterium.

1 31. The expression vector of claim 30, wherein the bacterium is *Escherichia coli*.

1 32. The expression vector of claim 14, wherein the host system comprises a  
2 eukaryotic cell.

1 33. The expression vector of claim 32, wherein the eukaryotic cell is a yeast.

1 34. The expression vector of claim 33, wherein the eukaryotic cell is a mammalian  
2 cell.

1 35. The expression vector of claim 14, wherein the host system comprises an  
2 archaeobacterium.

1 36. The expression vector of claim 14, wherein the host system comprises a plant  
2 cell.

1 37. The expression vector of claim 14, wherein the vector is a plasmid.

1 38. The expression vector of claim 14, wherein the vector is a bacteriophage.

1 39. The expression vector of claim 14, wherein the vector is a virus.

1 40. The expression vector of claim 14, wherein the vector is a linear nucleic acid  
2 molecule.

1 41. A substantially pure polypeptide comprising a first portion of a split intein, an  
2 second portion of a split intein, and a target peptide interposed between the first portion of a  
3 split intein and the second portion of a split intein, wherein the polypeptide is selected from  
4 the group consisting of: (a) a polypeptide that spontaneously splices in the host system to  
5 yield a cyclized form of the target peptide, and (b) a splicing intermediate of a cyclized form  
6 of the target peptide.

1 42. The polypeptide of claim 41, wherein the polypeptide is a polypeptide that  
2 spontaneously splices in the host system to yield a cyclized form of the target peptide.

1 43. The polypeptide of claim 41, wherein the polypeptide is a splicing  
2 intermediate of a cyclized form of the target peptide.

1 44. The polypeptide of claim 41, wherein the target peptide has a first end fused to  
2 the first portion of a split intein and a second end fused to the second portion of a split intein.

1 45. The polypeptide of claim 41, wherein both the first portion of a split intein and  
2 the second portion of a split intein are derived from a naturally-occurring split intein.

1 46. The polypeptide of claim 45, wherein both the first portion of a split intein and  
2 the second portion of a split intein are derived from Ssp DnaE.

1 47. The polypeptide of claim 41, wherein at least one of the first portion of a split  
2 intein and the second portion of a split intein is derived from a non-naturally occurring split  
3 intein.

1 48. The polypeptide of claim 47, wherein the non-naturally occurring split intein is  
2 derived from the group consisting of RecA, DnaB, Psp Pol-I, and Pfu inteins.

1 49. The polypeptide of claim 41, wherein both the first portion of a split intein  
2 and the second portion of a split intein are derived from a non-naturally occurring split intein.

1 50. The polypeptide of claim 43, wherein the splicing intermediate is a active  
2 intein intermediate.

1 51. The polypeptide of claim 43, wherein the splicing intermediate is a thioester  
2 intermediate.

1 52. The polypeptide of claim 43, wherein the splicing intermediate is a lariat  
2 intermediate.

1 53. A host system comprising a non-naturally occurring nucleic acid molecule  
2 encoding a polypeptide comprising a first portion of a split intein, a second portion of a split  
3 intein, and a target peptide interposed between the first portion of a split intein and the  
4 second portion of a split intein;  
5 wherein expression of the nucleic acid molecule in the host system produces the

- 6 polypeptide in a form selected from the group consisting of: (a) a polypeptide that  
7 spontaneously splices in the host system to yield a cyclized form of the target peptide, and (b)  
8 a splicing intermediate of a cyclized form of the target peptide.

1 54. The host system of claim 53, wherein the polypeptide is a polypeptide that  
2 spontaneously splices in the host system to yield a cyclized form of the target peptide.

1 55. The host system of claim 53, wherein the polypeptide is a splicing  
2 intermediate of a cyclized form of the target peptide.

56. The host system of claim 53, wherein the host system comprises a prokaryote.

57. The host system of claim 56, wherein the prokaryote is a bacterium.

58. The host system of claim 53, wherein the host system comprises an  
archaebacterium.

59. The host system of claim 53, wherein the host system comprises a eukaryote.

60. The host system of claim 59, wherein the eukaryote is a yeast.

61. The host system of claim 59, wherein the eukaryote is a mammalian cell.

62. The host system of claim 53, wherein the host system comprises a plant cell.

1           63.     A method for making a peptide molecule, the method comprising the steps of:  
2           providing an isolated nucleic acid molecule that encodes a polypeptide comprising a  
3     first portion of a split intein, a second portion of a split intein, and a target peptide interposed  
4     between the first portion of a split intein and the second portion of a split intein, wherein  
5     expression of the nucleic acid molecule in a host system produces the peptide molecule in a  
6     form selected from the group consisting of: (a) a cyclized form of the target peptide resulting  
7     from spontaneously splicing of the polypeptide in the host system, and (b) a splicing  
8     intermediate of a cyclized form of the target peptide;  
9           providing the host system;  
10          introducing the isolated nucleic acid molecule into the host system; and  
11          expressing the isolated nucleic acid molecule.

1           64.     The method of claim 63, wherein the step of expressing the isolated nucleic  
2     acid molecule results in production of a polypeptide that spontaneously splices in the host  
3     system to yield the cyclized form of the target peptide.

1           65.     The method of claim 64 further comprising the step of purifying the cyclized  
2     form of the target peptide from the host system.

1           66.     The method of claim 63, wherein the step of expressing the isolated nucleic  
2     acid molecule results in production of a splicing intermediate of a cyclized form of the target  
3     peptide.

1           67.     The method of claim 66 further comprising the step of purifying the splicing

2 intermediate of a cyclized form of the target peptide from the host system.

1 68. The method of claim 66, wherein the splicing intermediate is an active intein  
2 intermediate.

1 69. The method of claim 66, wherein the splicing intermediate is a thioester  
2 intermediate.

1 70. The method of claim 66, wherein the splicing intermediate is a lariat  
2 intermediate.

1 71. The method of claim 66, further comprising the step of forming the cyclic  
2 peptide from the splicing intermediate.

1 72. The method of claim 63, wherein the isolated nucleic acid molecule is  
2 incorporated into an expression vector that facilitates expression of the isolated nucleic acid  
3 molecule in the host system.

1 73. The method of claim 72, wherein the expression vector is a plasmid.

1 74. The method of claim 72, wherein the expression vector is a bacteriophage.

1 75. The method of claim 72, wherein the expression vector is a virus.



1 76. The method of claim 63, wherein the host system comprises a prokaryotic cell.

1 77. The method of claim 76, wherein the prokaryotic cell is a bacterium.

1 78. The method of claim 77, wherein the bacterium is *Escherichia coli*.

1 79. The host system of claim 63, wherein the host system comprises an  
2 archaeobacterium.

80. The method of claim 63, wherein the host system comprises a eukaryotic cell.

81. The method of claim 80, wherein the eukaryotic cell is a yeast.

82. The method of claim 80, wherein the eukaryotic cell is a mammalian cell.

1 83. The method of claim 63, wherein the host system comprises a plant cell.

1 84. The method of claim 63, wherein the host system comprises an in vitro  
2 transcription/translation system.

1 85. The method of claim 84, wherein the in vitro transcription/translation system  
2 comprises a cell lysate.

1 86. The method of claim 64, wherein the production of the target peptide in

2 cyclized form occurs in the host system in the absence of an exogenously-added agent.

1 87. The method of claim 86, wherein the exogenously-added agent is a protease.

1 88. The method of claim 86, wherein the exogenously-added agent is a thiol.

1 89. The method of claim 72, wherein the expression vector is inducible.

1 90. A method of preparing a library of peptide molecules, the method comprising  
2 the steps of:

3 providing a plurality of nucleic acid molecules encoding a plurality of target peptides  
4 having heterogenous amino acid sequences;

5 incorporating each of the plurality of nucleic acid molecules into an expression vector  
6 to form a plurality of expression vectors, whereby each of the plurality of nucleic acid  
7 molecules is interposed between a nucleic acid molecule encoding a first portion of a split  
8 intein and a nucleic acid molecule encoding an second portion of a split intein in each of the  
9 formed expression vectors, wherein expression of the expression vectors in a host system  
10 results in the production of a plurality of peptide molecules in a form selected from the group  
11 consisting of: (a) polypeptides that spontaneously splice in the host system to yield cyclized  
12 forms of the target peptides, and (b) splicing intermediates of cyclized forms of the target  
13 peptides; and

14 expressing the expression vectors in the host system.

1 91. The method of claim 90, wherein the plurality of polypeptides are polypeptides

2 that spontaneously splice in the host system to yield cyclized forms of the target peptides.

1 92. The method of claim 90, wherein the plurality of polypeptides are splicing  
2 intermediates of cyclized forms of the target peptides

1 93. The method of claim 90, wherein the plurality of nucleic acid molecules  
2 encoding a plurality of target peptides are produced by solid phase synthesis.

1 94. The method of claim 90, wherein the plurality of nucleic acid molecules  
2 encoding a plurality of target peptides are produced using polymerase chain reaction.

1 95. The method of claim 90, wherein the plurality of nucleic acid molecules  
2 encoding a plurality of target peptides are produced by enzymatically digesting a larger  
3 nucleic acid molecule.

1 96. The method of claim 95, wherein the larger nucleic acid molecule is derived  
2 from an organism.

1 97. The method of claim 90, wherein the plurality of nucleic acid molecules  
2 encoding a plurality of target peptides are produced from a progenitor nucleic acid molecule  
3 that has been amplified under conditions which introduce mutations into the progenitor  
4 nucleic acid molecule's nucleotide sequence.

1 98. A method of screening a peptide molecule for a predetermined characteristic,

the method comprising the steps of:

providing a nucleic acid molecule that encodes a polypeptide comprising a first portion of a split intein, a second portion of a split intein, and a target peptide interposed between the first portion of a split intein and the second portion of a split intein, wherein expression of the nucleic acid molecule in a host system produces the peptide molecule in a form selected from the group consisting of: (a) a cyclized form of the target peptide resulting from spontaneously splicing of the polypeptide in the host system, and (b) a splicing intermediate of a cyclized form of the target peptide;

providing the host system;

introducing the isolated nucleic acid molecule in the host system;

placing the host system under conditions that cause the peptide molecule to be produced; and

testing the peptide molecule for the predetermined characteristic.

99. The method of claim 98, wherein the peptide molecule is a cyclized form of the target peptide.

100. The method of claim 98, wherein the peptide molecule is a splicing intermediate of a cyclized form of the target peptide.

101. The method of claim 98, wherein the predetermined characteristic comprises the ability to specifically bind a target molecule, and the step of testing the peptide molecule for the predetermined characteristic comprises the steps of (a) contacting the peptide molecule to the target molecule and (b) determining whether the peptide molecule binds to

5 the target molecule.

1 102. The method of claim 101, wherein the step of determining whether the peptide  
2 molecule binds to the target molecule is measured by observing a color change.

1 103. The method of claim 101, wherein the step of determining whether the peptide  
2 molecule binds to the target molecule is measured by observing a fluorescent signal.

1 104. The method of claim 101, wherein the step of determining whether the peptide  
2 molecule binds to the target molecule is measured by analyzing the cell cycle of an organism.

1 105. The method of claim 101, wherein the step of determining whether the peptide  
2 molecule binds to the target molecule is measured by analyzing the reproduction of an  
3 organism.

1 106 The method of claim 101, wherein the target molecule is a cell-associated  
2 molecule.

1 107. The method of claim 106, wherein the cell-associated molecule is a  
2 membrane-associated molecule.

1 108. The method of claim 106, wherein the cell-associated molecule is an  
2 intracellular molecule.

1           109.    The method of claim 108, wherein the intracellular molecule is a nuclear  
2 molecule.

1           110.    The method of claim 108, wherein the intracellular molecule is an organelle.

1           111.    The method of claim 110, wherein the organelle is selected from the group  
2 consisting of: mitochondria, lysosomes, endoplasmic reticula, chloroplasts, golgi, and  
3 periplasm.

1           112.    The method of claim 101, wherein the target molecule is an extracellular  
2 molecule.

1           113.    The method of claim 98, wherein the predetermined characteristic is the ability  
2 to modulate a biochemical reaction, and the step of testing the peptide molecule for the  
3 predetermined characteristic comprises the steps of (a) contacting the peptide molecule to a  
4 system containing the biochemical reaction and (b) determining whether the peptide molecule  
5 modulates the biochemical reaction.

1           114.    The method of claim 113, wherein the step of determining whether the peptide  
2 molecule modulates the biochemical reaction is measured by observing a color change.

1           115.    The method of claim 113, wherein the step of determining whether the peptide  
2 molecule modulates the biochemical reaction is measured by observing a fluorescent signal.

1 116. The method of claim 113, wherein the step of determining whether the peptide  
2 molecule modulates the biochemical reaction is measured by analyzing the cell cycle of an  
3 organism.

1 117. The method of claim 113, wherein the step of determining whether the peptide  
2 molecule modulates the biochemical reaction is measured by analyzing the reproduction of an  
3 organism.

1 118. The method of claim 113, wherein the biochemical reaction is an a cell-  
associated process. 119. The method of claim 118, wherein the biochemical reaction is  
2 an intracellular metabolic event.

1 120. The method of claim 118, wherein the biochemical reaction is a membrane-  
2 associated event.

1 121. The method of claim 118, wherein the biochemical reaction is a nuclear event.

1 122. The method of claim 113, wherein the biochemical reaction is a extracellular  
2 reaction.

1 123. The method of claim 98, wherein the step of testing the peptide molecule for  
2 the predetermined characteristic is performed using a hybrid system.

1 124. The method of claim 98, further comprising the step of immobilizing the

2 peptide molecule on a solid phase support.

1 125. A method for purifying a cyclic peptide from a mixture, the method

2 comprising the steps of:

3 providing a mixture containing a splicing intermediate conjugated with an affinity tag;

4 mixing the conjugated splicing intermediate with a solid phase support having a

5 ligand thereon that specifically binds the affinity tag whereby the support becomes

6 specifically bound with the splicing intermediate;

7 washing the support to remove non-specifically bound matter from the support;

8 adding to the support a reagent that makes a cyclic peptide from the splicing

9 intermediate; and

10 eluting the cyclic peptide from the support.

126. A method for purifying a cyclic peptide from a mixture, the method

comprising the steps of:

providing a mixture containing a splicing intermediate conjugated with an affinity tag;

mixing the conjugated splicing intermediate with a solid phase support having a

ligand thereon that specifically binds the affinity tag whereby the support becomes

specifically bound with the splicing intermediate;

washing the support to remove non-specifically bound matter from the support;

eluting the splicing intermediate from the support; and

adding a reagent the eluted splicing intermediate that make a cyclic peptide from  
the splicing intermediate;



- 1           127. A method for purifying a target molecule that binds a splicing intermediate  
2 from a mixture, the method comprising the steps of:  
3           providing a solid phase support having the splicing intermediate specifically bound  
4 thereon;  
5           contacting the support with the target molecule in the mixture;  
6           washing the support to remove non-specifically bound matter from the support; and  
7           eluting the target molecule from the support.



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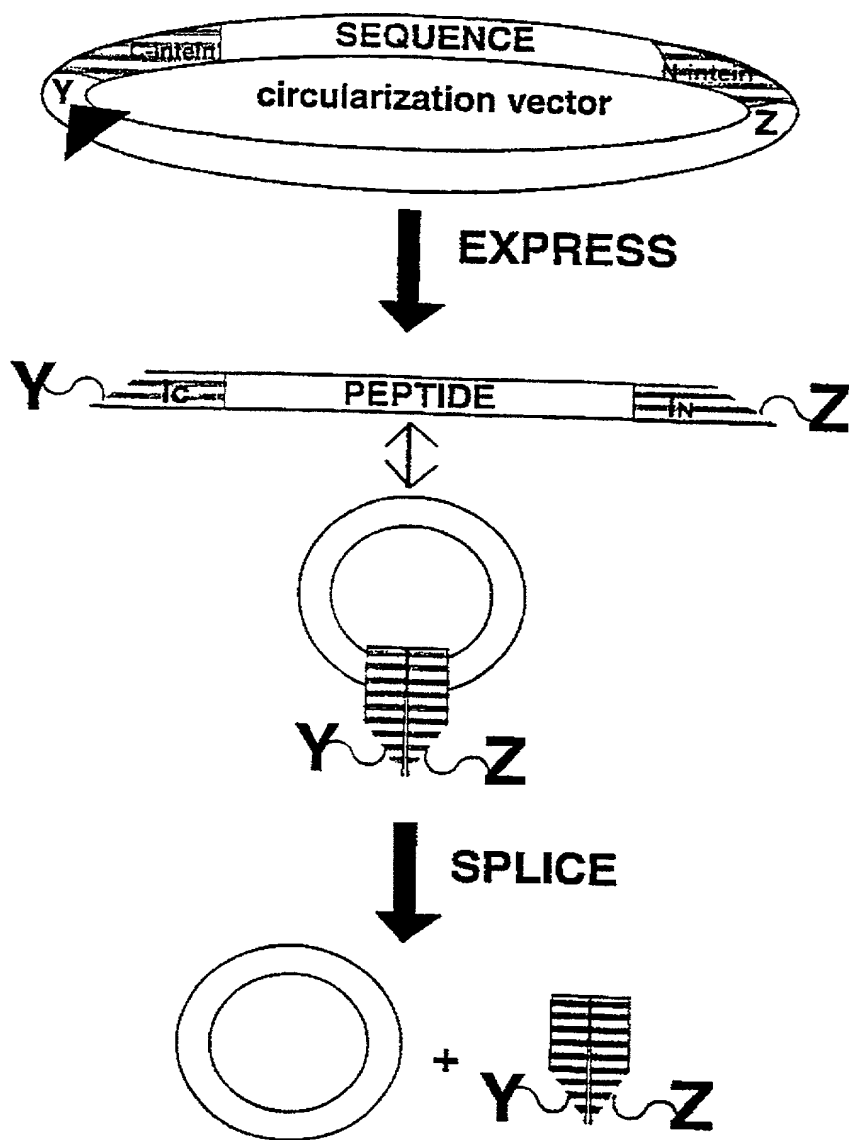


FIG. 1

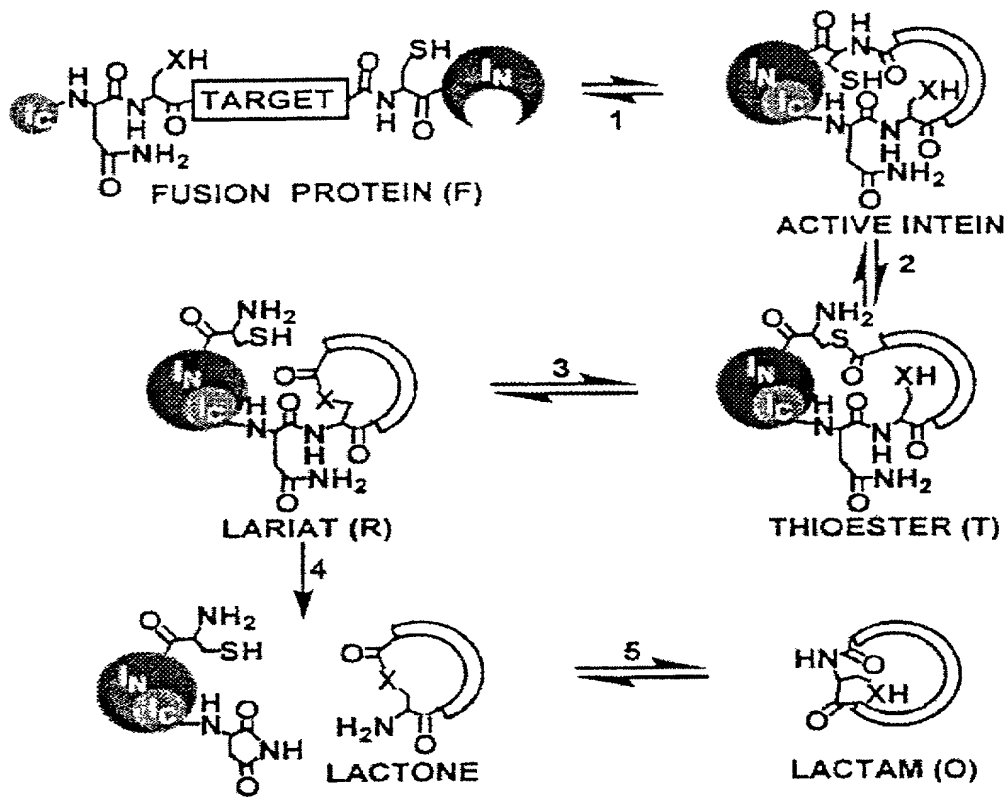


FIG. 2

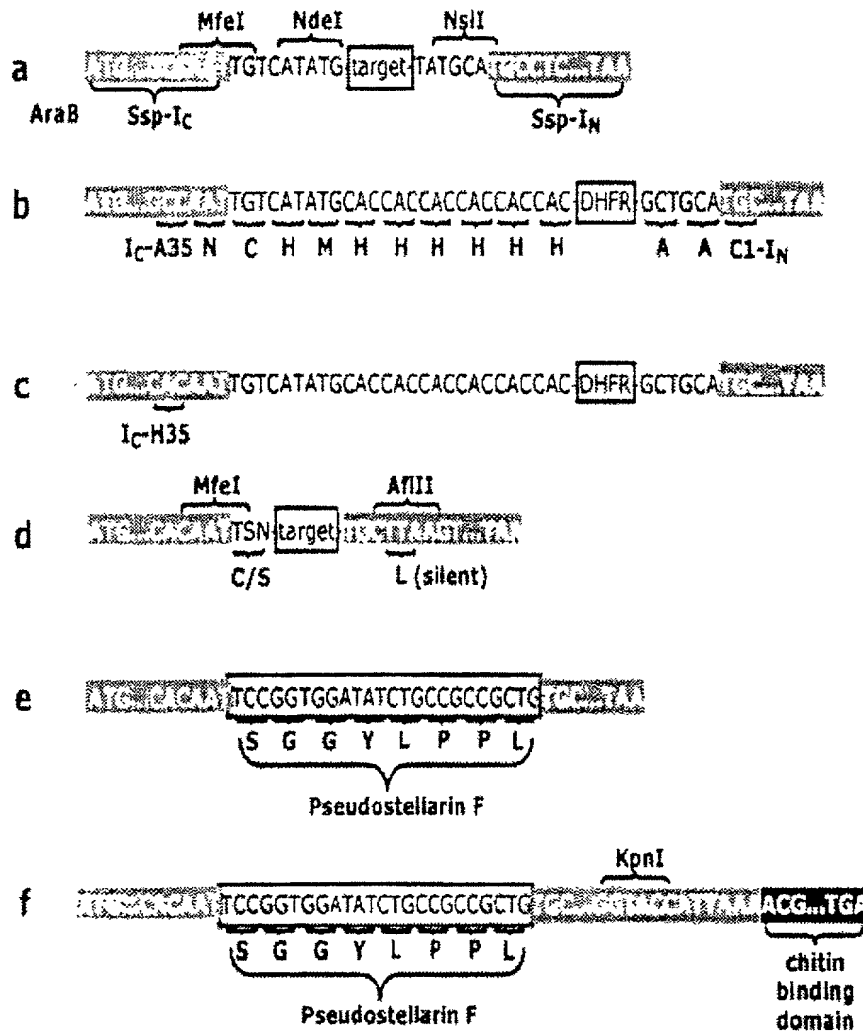


FIG. 3

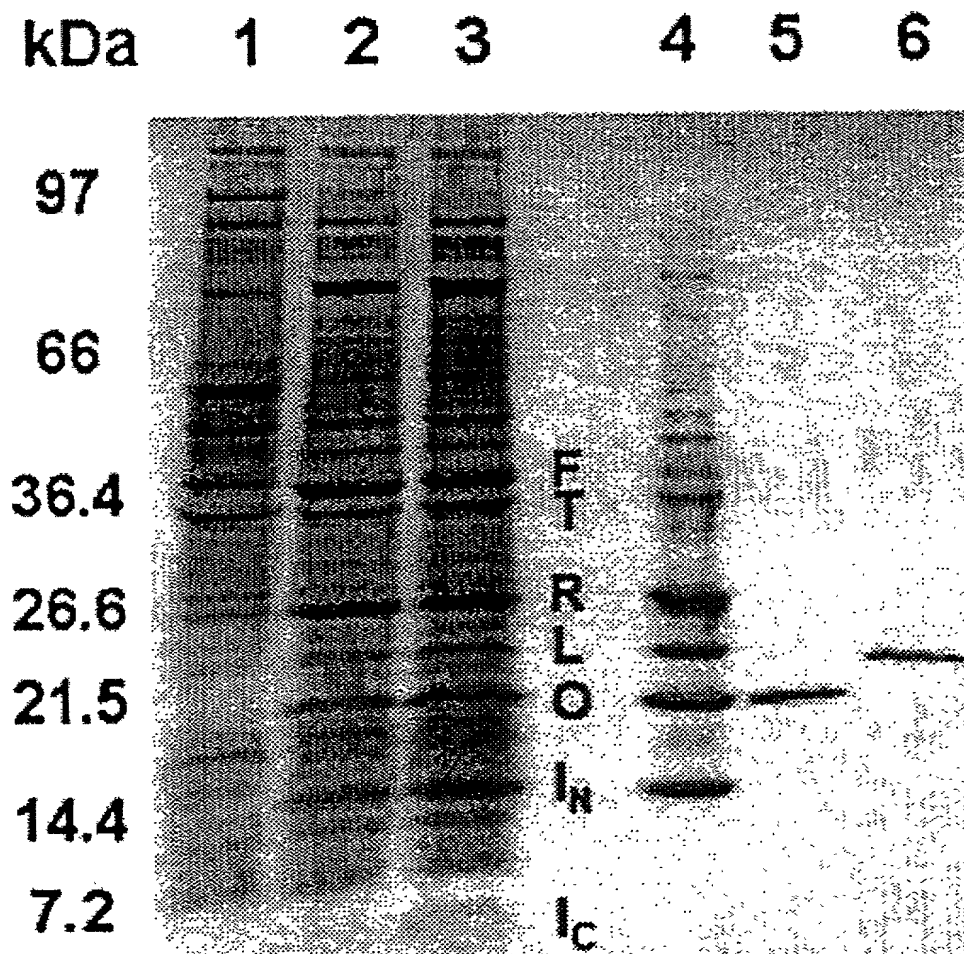


FIG. 4

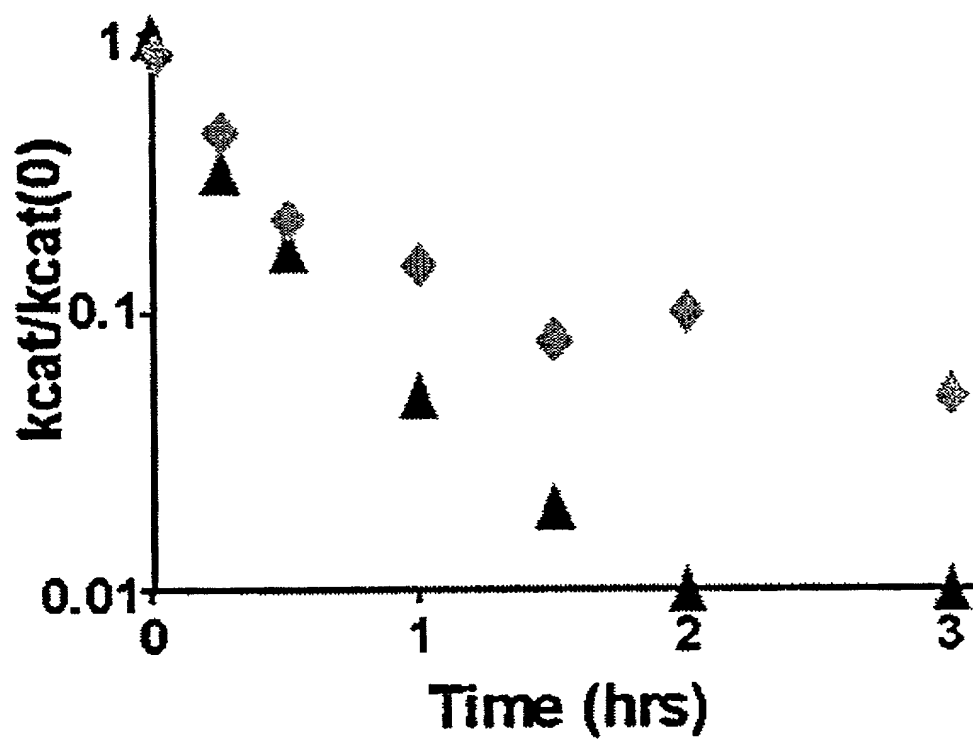


FIG. 5

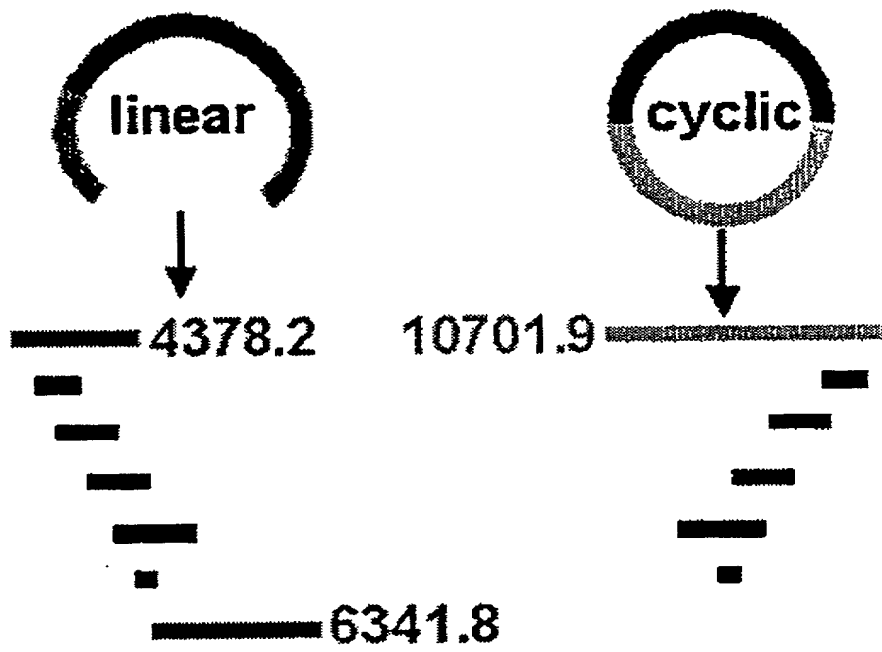


FIG. 6



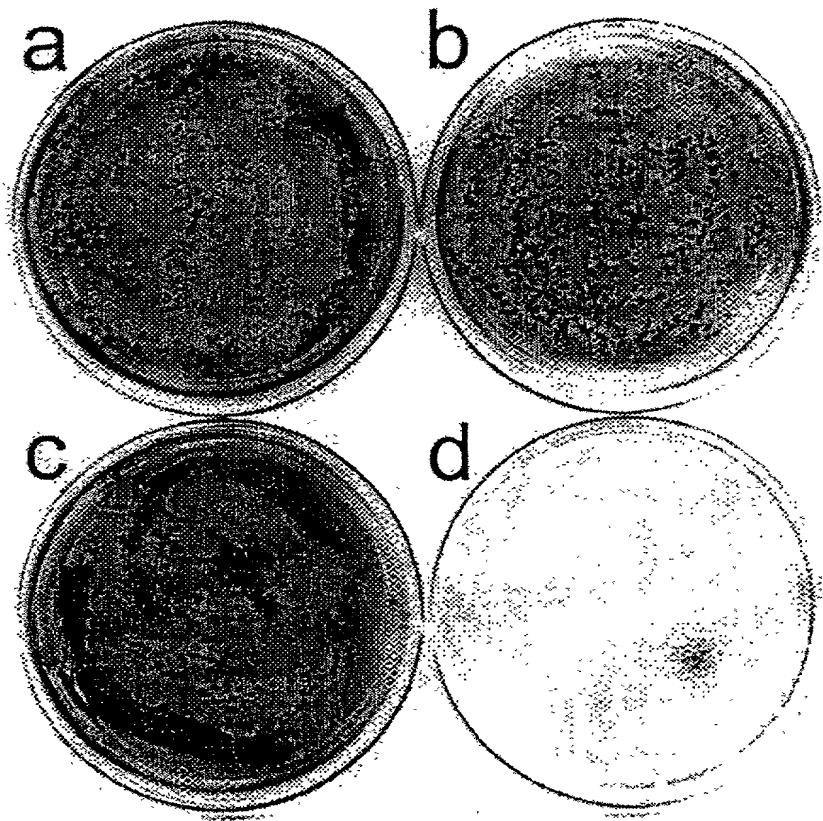


FIG. 7

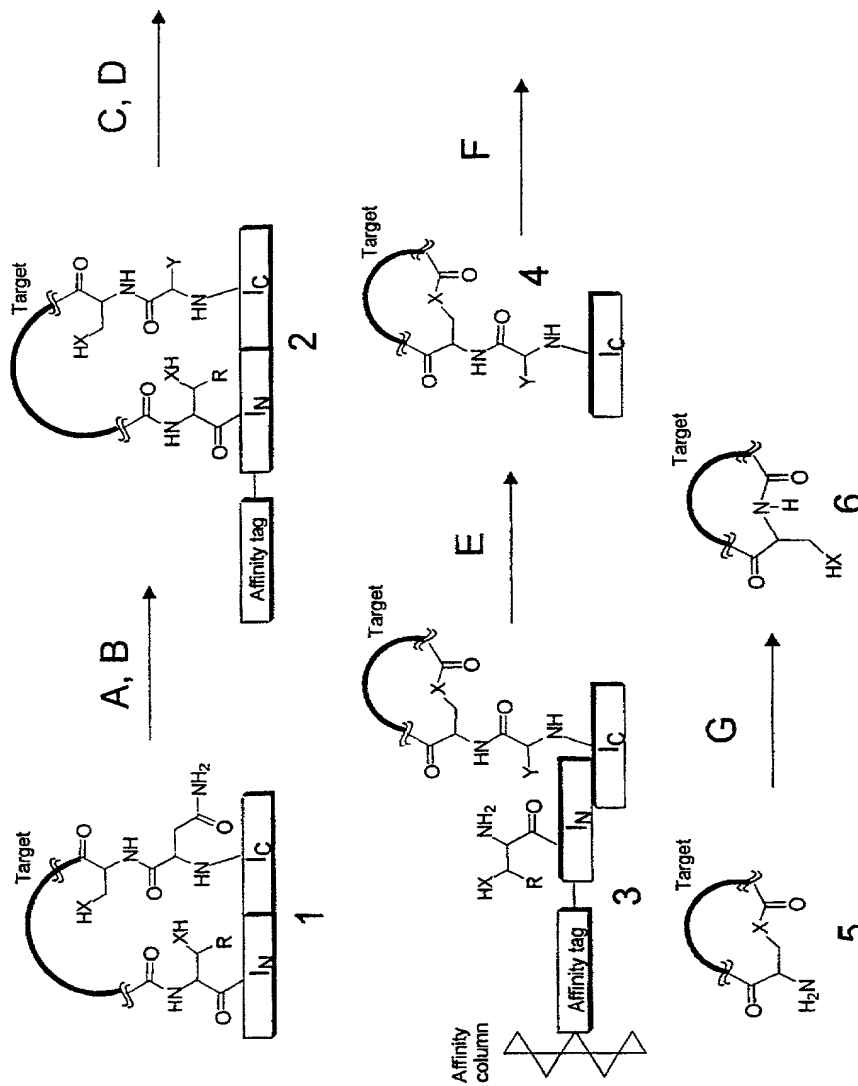


FIG. 8

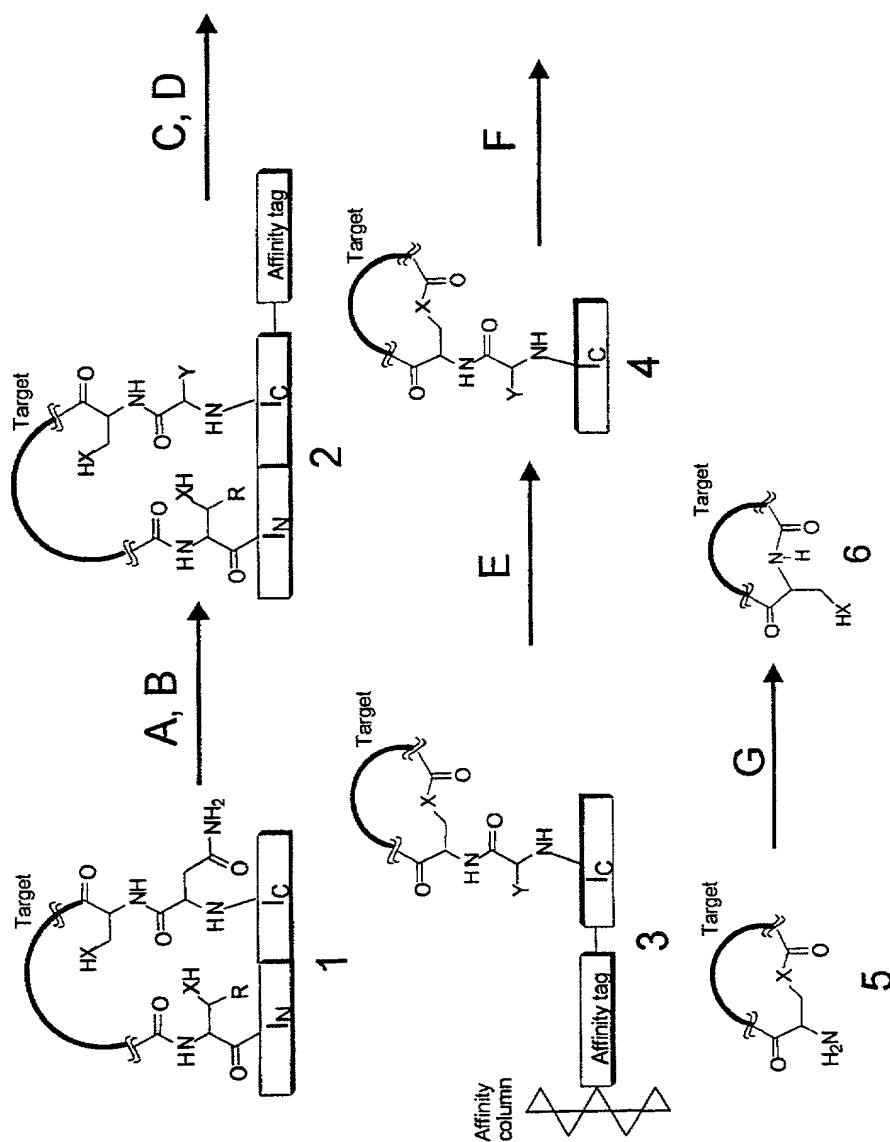


FIG. 9

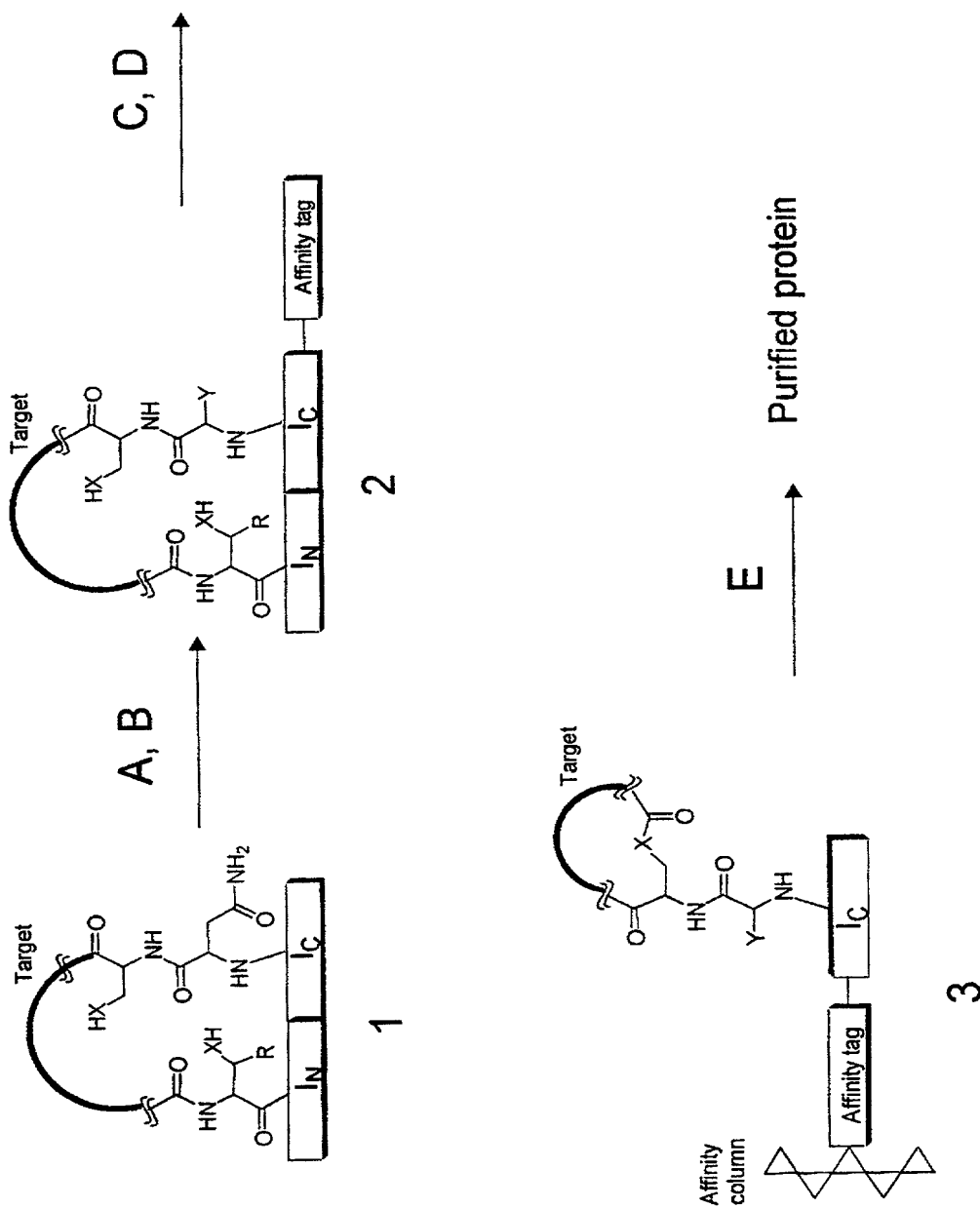


FIG. 10

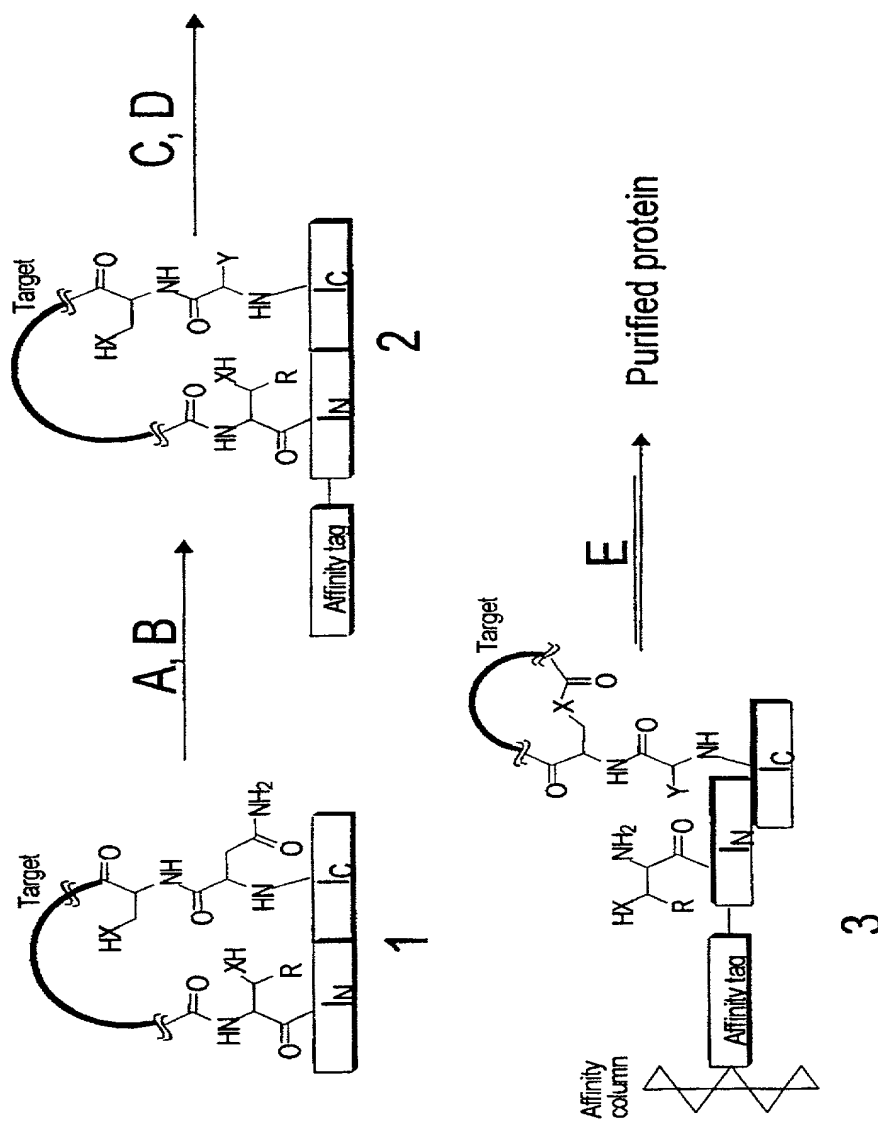


FIG. 11

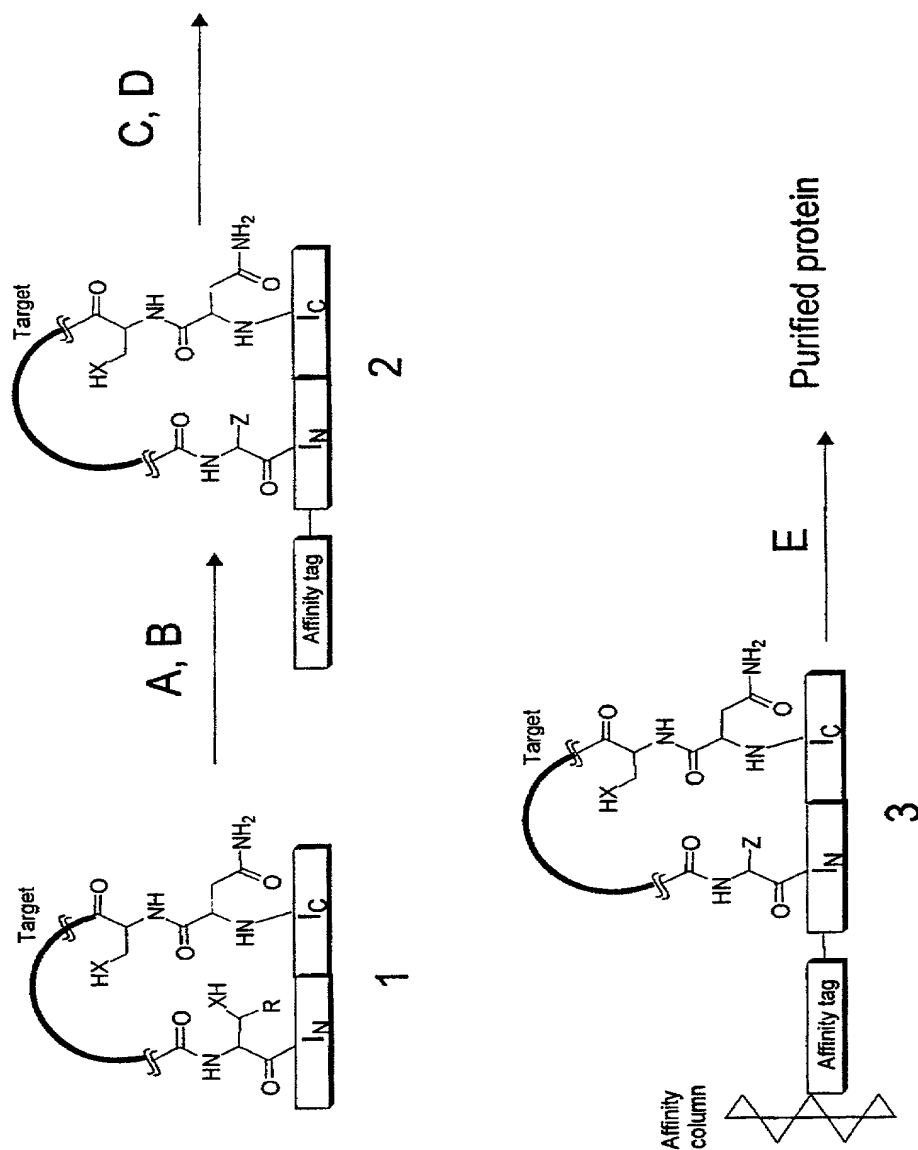


FIG. 12

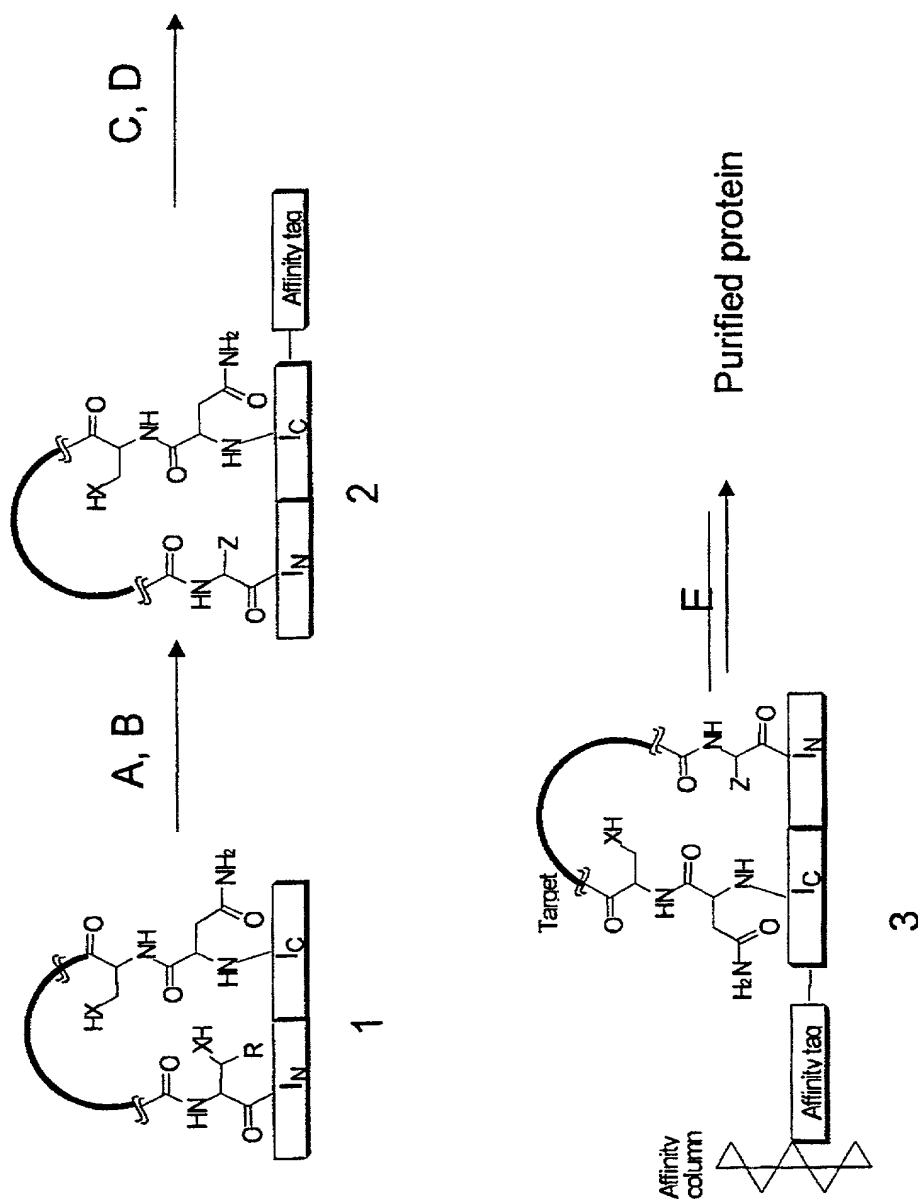


FIG. 13

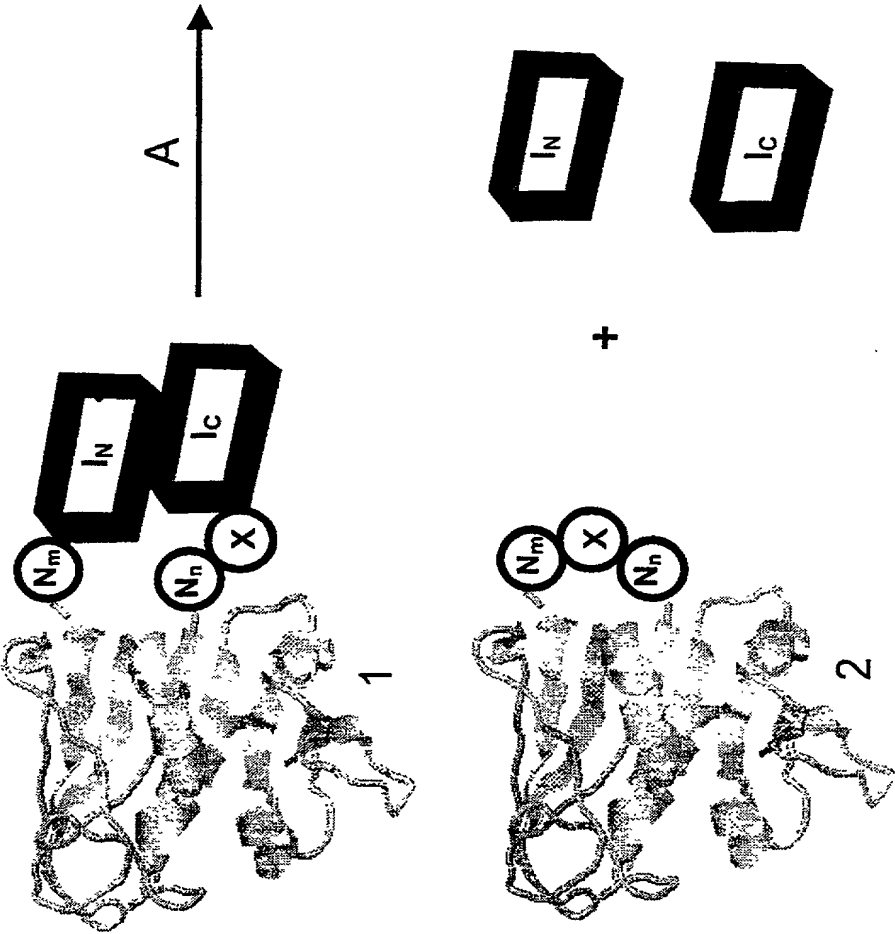


FIG. 14



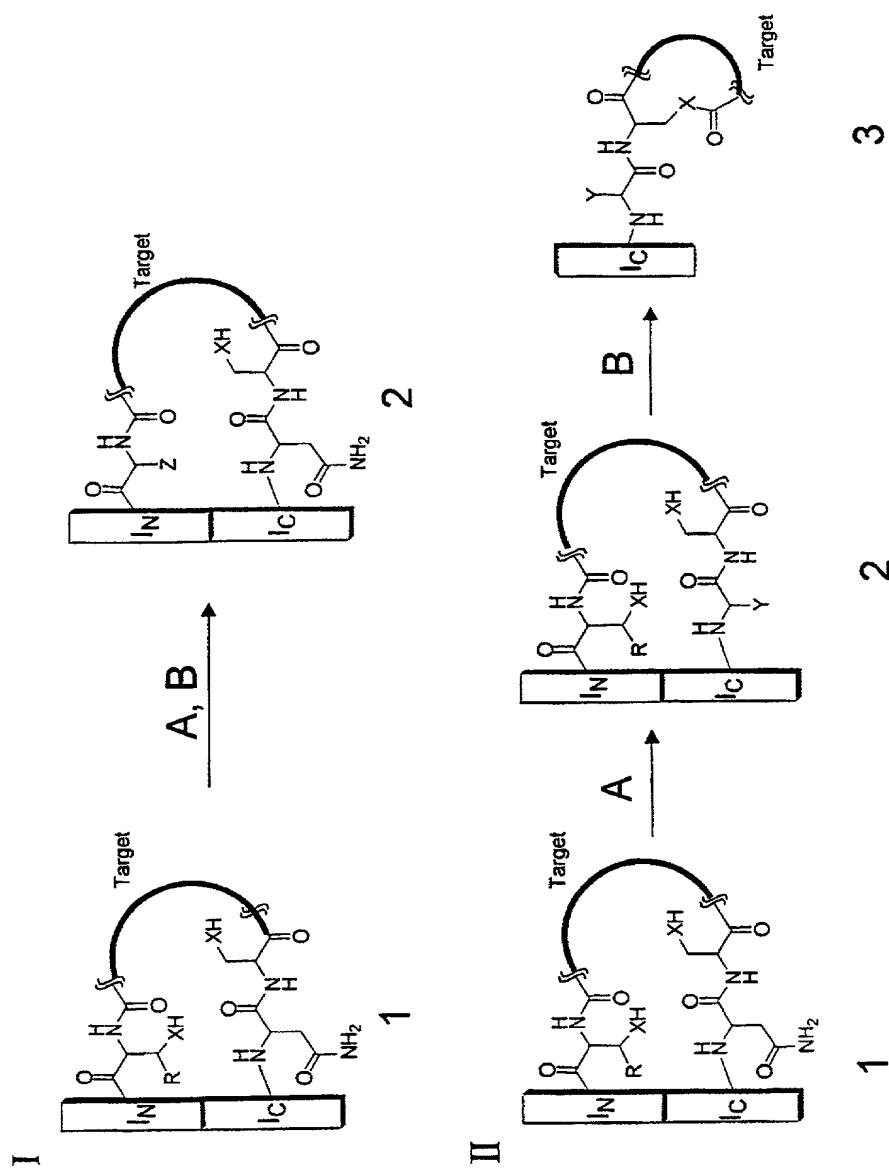


FIG. 15

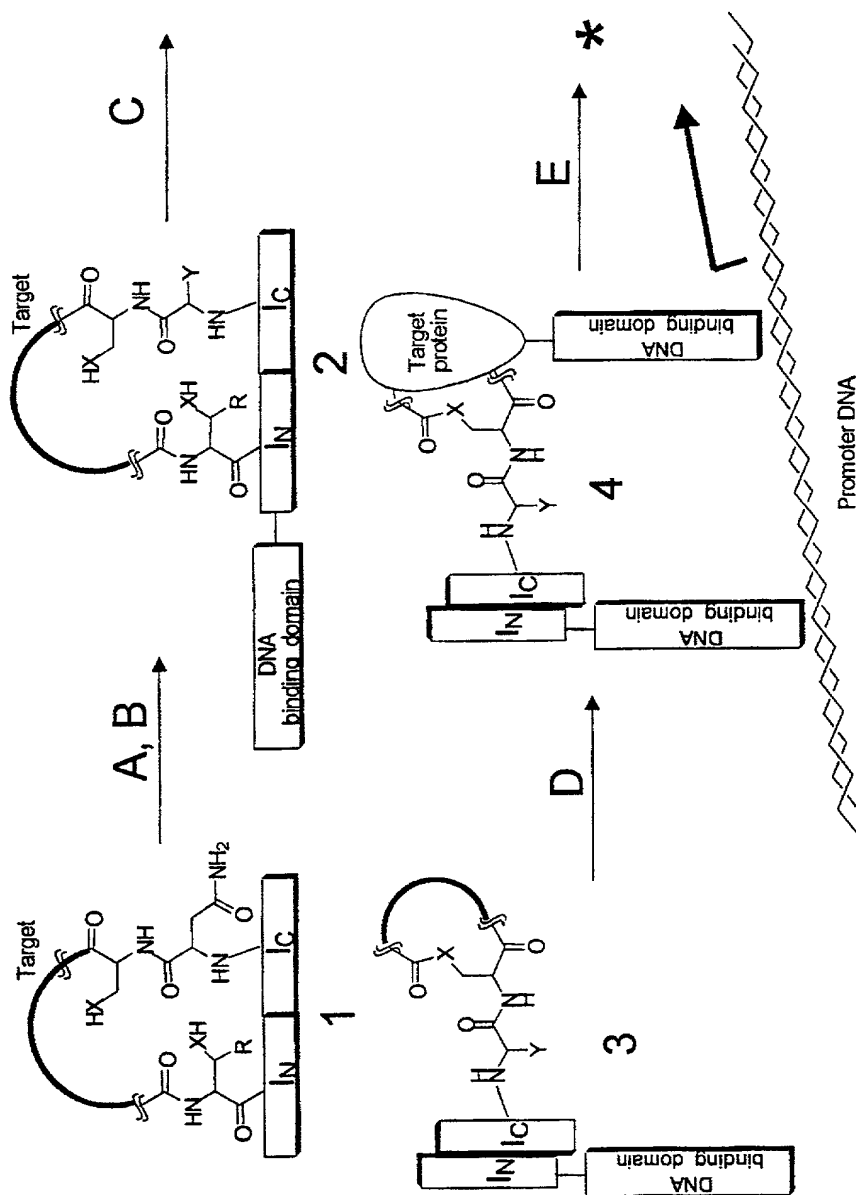


FIG. 16

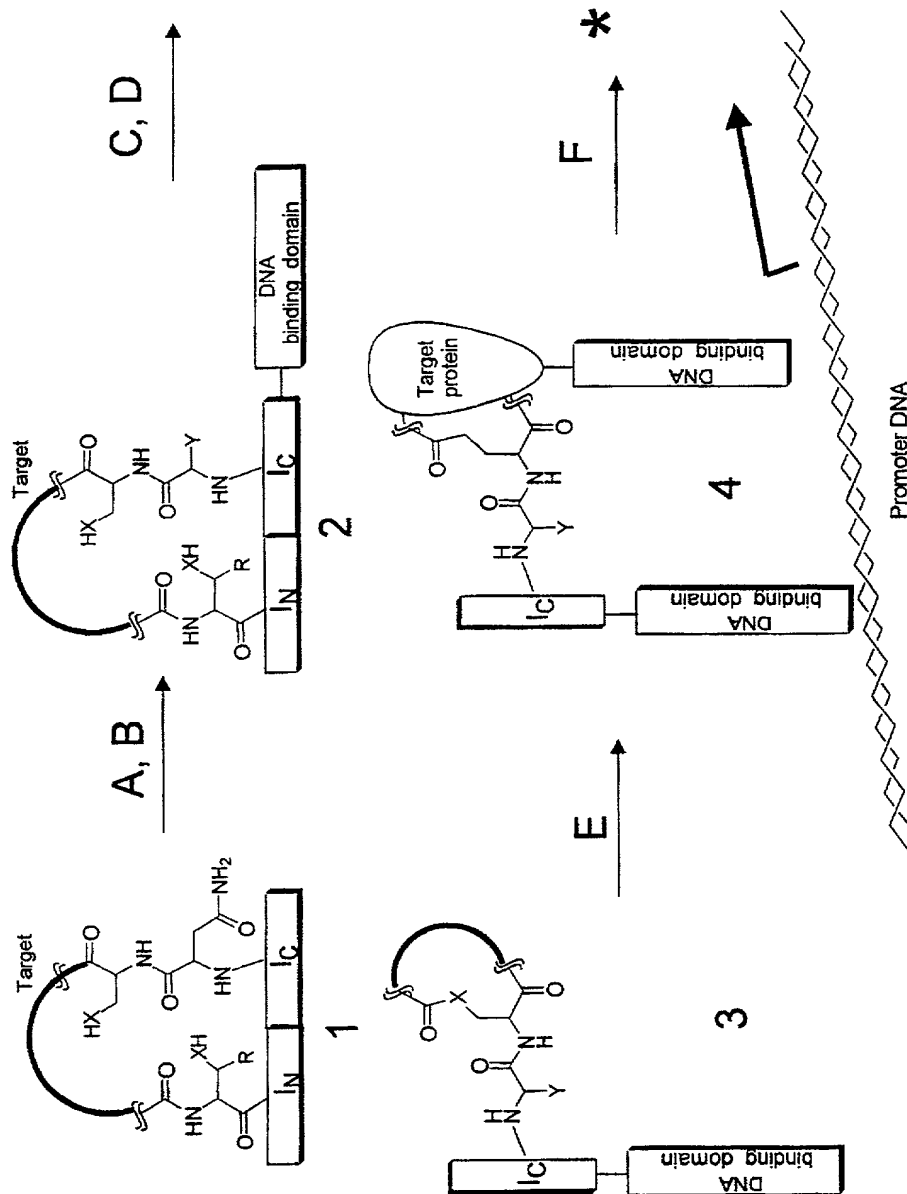


FIG. 17

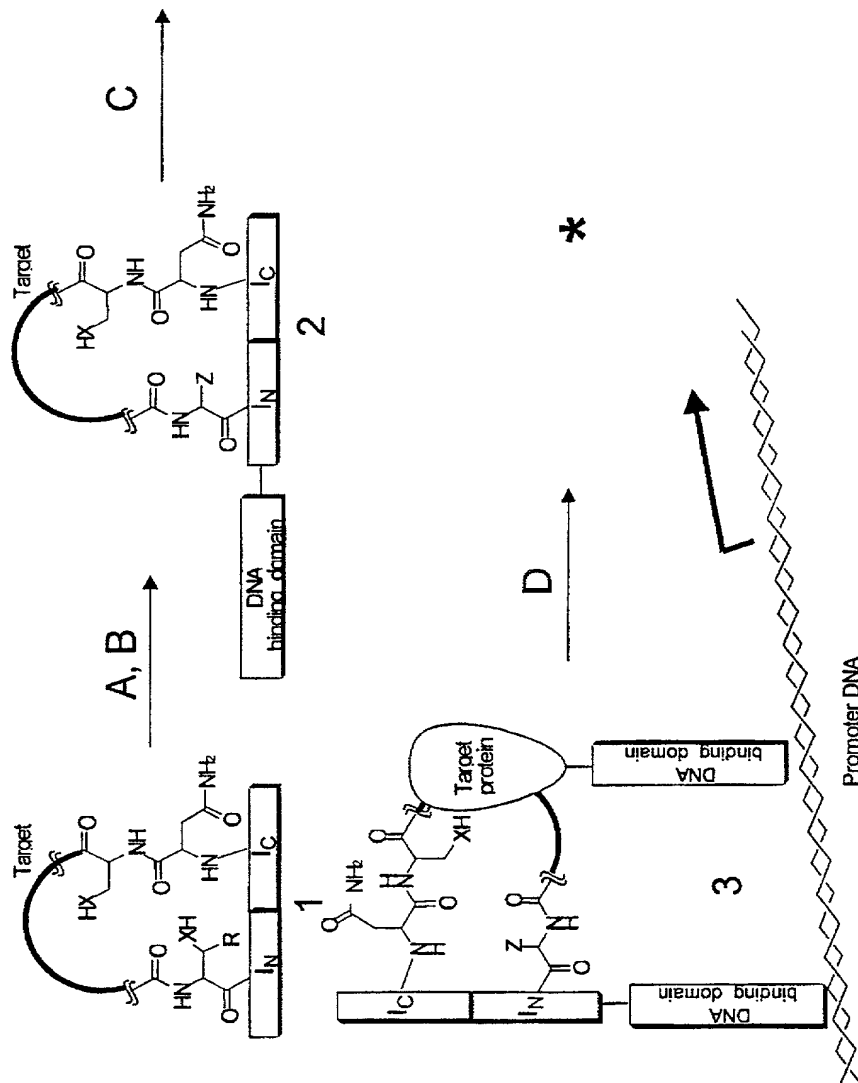


FIG. 18

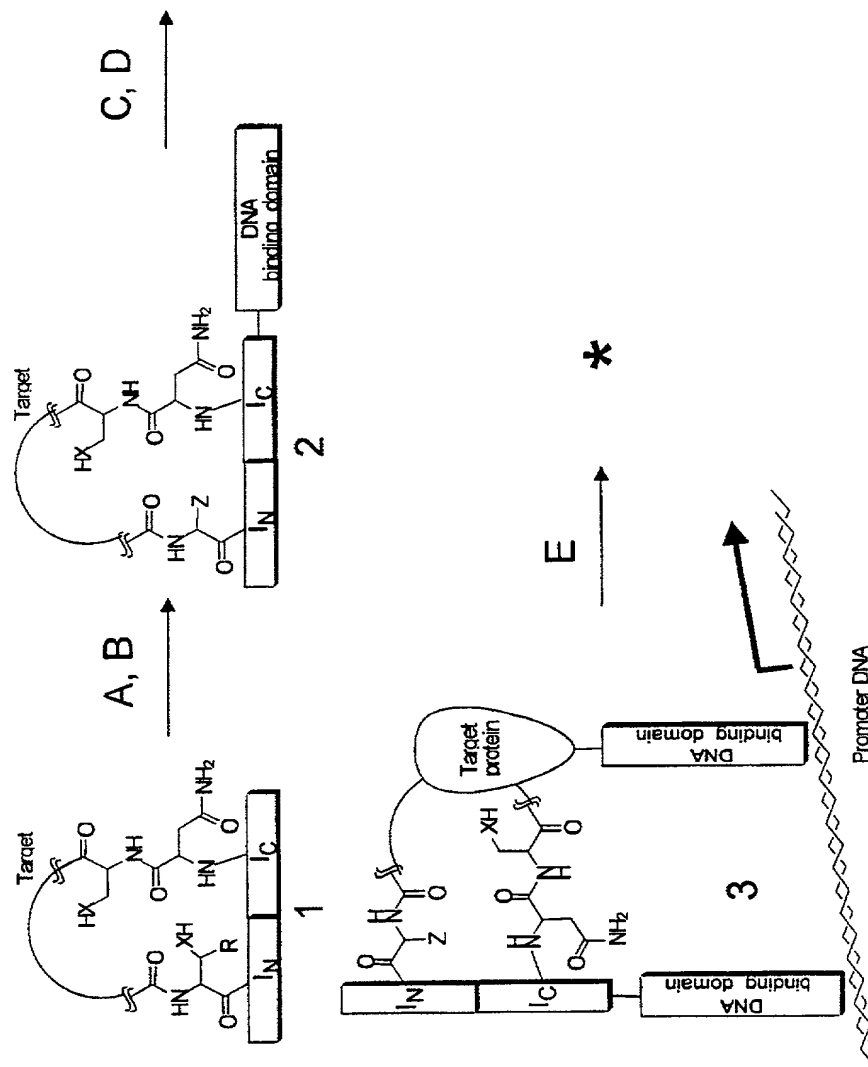


FIG. 19

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Cyclic peptides

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as U.S. Patent Application Serial Number \_\_  
on \_\_,  
as amended on \_\_ (if applicable).

☒ was filed as a PCT international application number PCT/US99/30162 on 18  
December 1999, as amended under PCT Article 19 on \_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
US	60/112,723	18 December 1998	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
US	60/158,077	7 October 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

**COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**  
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER  
**6460-18-1**

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:**

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.		
PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS
PCT/US99/30162	18 December 1999	

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith: J. Rodman Steele, Jr., Registration No. 25,931; Gregory A. Nelson, Registration No. 30,577, Joseph W. Bain, Registration No. 34,290; Robert J. Sacco, Registration No. 35,667; Mark D. Passler, Registration No. 40,764; Stanley A. Kim, Registration No. 42,730; Steven M. Greenberg, Registration No. 44,725; Neil R. Jetter, Registration No. 46,803; Larry G. Brown, Registration No. 45,834; Kevin T. Cuenot, Registration No. 46,283; Pablo Meles, Registration No. 33,739; Raynardo K. Whitty, Registration No. 47,176; Barbara S. Kitchell, Registration No. 33,928; and Terry Forsythe, Registration No. 47,569

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West Palm Beach, FL 33402-3188

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(561) 653-5000

201	FULL NAME OF INVENTOR	FAMILY NAME <u>BENKOVIC</u>	FIRST GIVEN NAME <u>STEPHEN</u>	SECOND GIVEN NAME <u>J.</u>
	RESIDENCE & CITIZENSHIP	CITY <u>STATE COLLEGE</u> <i>PA.</i>	STATE OR COUNTRY <u>PA</u>	COUNTRY OF CITIZENSHIP <u>US</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>771 TEABERRY LANE</u>	CITY <u>STATE COLLEGE</u>	STATE & ZIP CODE/COUNTRY <u>PA 16801</u>
202	FULL NAME OF INVENTOR	FAMILY NAME <u>SCOTT</u>	FIRST GIVEN NAME <u>CHARLES</u>	SECOND GIVEN NAME <u>P.</u>
	RESIDENCE & CITIZENSHIP	CITY <u>STATE COLLEGE</u> <i>PA.</i>	STATE OR COUNTRY <u>PA</u>	COUNTRY OF CITIZENSHIP <u>US</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>1614 HIGHLANDON COURT</u>	CITY <u>STATE COLLEGE</u>	STATE & ZIP CODE/COUNTRY <u>PA 16801</u>
203	FULL NAME OF INVENTOR	FAMILY NAME <u>ABEL-SANTOS</u>	FIRST GIVEN NAME <u>ERNESTO</u>	SECOND GIVEN NAME <u>V.</u>
	RESIDENCE & CITIZENSHIP	CITY <u>STATE COLLEGE</u> <i>PA.</i>	STATE OR COUNTRY <u>PA</u>	COUNTRY OF CITIZENSHIP <u>US</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>1635 HIGHLANDON COURT</u>	CITY <u>STATE COLLEGE</u>	STATE & ZIP CODE/COUNTRY <u>PA 16801</u>

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <i>[Signature]</i>	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE <i>8-23-'01</i>	DATE	DATE

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2-00

3-00

**COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**  
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER  
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**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:**

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.			
PCT APPLICATION NUMBER	PCT FILING DATE	U S. SERIAL NUMBERS	
PCT/US99/30162	18 December 1999		


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201	FULL NAME OF INVENTOR	FAMILY NAME BENKOVIC	FIRST GIVEN NAME STEPHEN	SECOND GIVEN NAME J.
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	POST OFFICE ADDRESS	POST OFFICE ADDRESS 771 TEABERRY LANE	CITY STATE COLLEGE	STATE & ZIP CODE/COUNTRY PA 16801
202	FULL NAME OF INVENTOR	FAMILY NAME SCOTT	FIRST GIVEN NAME CHARLES	SECOND GIVEN NAME P.
	RESIDENCE & CITIZENSHIP	CITY STATE COLLEGE	STATE OR COUNTRY PA	COUNTRY OF CITIZENSHIP US
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 1614 HIGHLANDON COURT	CITY STATE COLLEGE	STATE & ZIP CODE/COUNTRY PA 16801
203	FULL NAME OF INVENTOR	FAMILY NAME ABEL-SANTOS	FIRST GIVEN NAME ERNESTO	SECOND GIVEN NAME V.
	RESIDENCE & CITIZENSHIP	CITY STATE COLLEGE	STATE OR COUNTRY PA	COUNTRY OF CITIZENSHIP US
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 1635 HIGHLANDON COURT	CITY STATE COLLEGE	STATE & ZIP CODE/COUNTRY PA 16801

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202 	SIGNATURE OF INVENTOR 203
DATE	DATE 9/27/2001	DATE

T04350 "6948860



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY  
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER  
6460-18-1

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PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS		
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	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
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	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE	DATE	DATE

CPS